The Potential for Localised Inhibition of Complement and Coagulation Cascades in High Risk Renal Transplantation

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The Potential for Localised Inhibition of Complement and Coagulation Cascades in High Risk Renal Transplantation

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Abstract

Transplantation is the preferred treatment for patients with end-stage kidney disease. However, antibodies against blood group and histocompatibility antigens on transplanted organs pose a high risk of accelerated humoral rejection. Allograft transplantation into sensitised recipients initiates complement activation and early thrombotic processes. The aim of the research described here was to determine whether cell protective therapy targeting the complement and coagulation cascades is effective at preventing graft rejection in highly sensitised renal allograft recipients.

In this thesis, using novel membrane targeting (cytotopic) technology, a therapeutic complement regulator Mirococept (APT070) and coagulation inhibitors (PTL006, Thrombalexin/PTL004) were delivered to the endothelial surface within organs. In these studies, a rat model of hyperimmune rejection of renal allografts from fully MHC disparate donors and recipients was established. *In vivo* studies revealed the potential of the therapeutic reagents by assessment of graft survival, renal function and pathology.

Mirococept only provided modest protection of the endothelium and in renal function, without any prolongation of survival compared to recipients of control-treated grafts. In contrast, treatment of kidneys with either anticoagulant agent (PTL006 or Thrombalexin/PTL004) alone resulted in significant prolongation of graft survival correlating with delayed loss of renal function compared to controls. To explore whether these findings were suitable for translation to clinical application, the investigations were extended to include standard immunosuppressive agents. However, Cyclosporine A treatment was associated with toxic effects in the rats, whereas therapy including Rapamycin failed to prevent lymphocyte migration into the grafts.

This study provides evidence that *ex vivo* graft perfusion of locally acting biological response modifiers can have therapeutic effects in the transplant setting. The approach targets donor organs rather than the recipient. Additional and effective inhibition of cellular immune responses may demonstrate the full potential of this approach to attain graft acceptance against the most severe of immune barriers. These findings make this study clinically relevant.

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Abbreviations

α1.3GT	1.3 Galactosyltransferase
AAMR	Acute Antibody-Mediated Rejection
ABO	ABO Blood Group Antigens
ADCC	Antibody-Dependent Cellular Cytotoxicity
AECAs	Anti-Endothelial Cell Antibodies
AHR	Acute Humoral Rejection
AMR	Antibody-Mediated Rejection
AP	Alternative Pathway
APC	Activating Protein C
ASC	Antigen Secreting Cells
AT-III	Antithrombin-III
ATM	Acute Thrombotic Microangiopathy
AZA	Azathioprine
BCR	B-Cell Receptor
BSA	Bovine Serum Albumin
C4BP	C4b-Binding Protein
CAMR	Chronic Antibody-Mediated Rejection
CHO	Chinese Hamster Ovary
CP	Classical Pathway
CPHAD	Classical Pathway Haemolytic Diluents Buffer
CR1	Complement Receptor 1
CRD	Carbohydrate Recognition Domains
CRP	C-Reactive Protein
CsA	Cyclosporine A
CSR	Class Switch Recombination
DA	Dark Agouti
DAMPs	Damage-Associated Molecular Patterns
DMSO	Dimethyl Sulphoxide
DSA	Donor-Specific Antibody
DTH	Delayed Type Hypersensitivity
ECs	Endothelial Cells
ENDATS	Endothelial Cell-Associated Transcripts

FCS	Foetal Calf Serum
FCRs	FcReceptors
FGF	Fibroblast Growth Factor
fI	factor I
FK-506	Tacrolimus
Galα1, 3Gal	Galactose-α-1,3 Galactose
GBM	Glomerular Basement Membrane
HLA	Human Leukocyte Antigen
HMWK	High Molecular Weight Kininogen
ICAM	intercellular adhesion molecule-1
Ig	Immunoglobulin
LHR	Long Homologous Repeat
LP	Lectin Pathway
MAC	Membrane Attack Complex
MASP	MBL-associated serine protease
MBL	Mannose Binding Lectin
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MiHA	Minor Histocompatibility Antigen
MMF	Mycophenolate Mofetil
MSB	Martius Scarlet Blue
NO	Nitric Oxide
OCT	Optimal Cutting Temperature
PAMPs	Pathogen Associated Molecular Patterns
PARs	Protease Activated Receptors
PBS	Phosphate Buffer Saline
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear
PGI_2	Prostacyclin
PK	Prekallikrein
PTC	Peritubular Capillaries

RBC	Red Blood Cells
RT1	Major Histocompatibility Complex of the rat
SCRs	Short Consensus Repeats
SD	Sprague Dawley
SHM	Somatic Hypermutation
SPI	Solid Phase Immunoassay
TAFI	Thrombin-Activatable Fibrinolysis Inhibitor
TCC	Terminal Complement Complex
TCRs	T-Cell Receptors
T-D	T-Cell Dependent
TF	Tissue Factor
T-I	T-Cell Independent
TLRs	Toll-Like Receptors
t-PA	tissue-Plasminogen Activator
TXA_2	Thromboxane A_2
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor

Chapter 1 – Introduction

1.1 The Kidney

1.1.1 Function of the Kidney

The kidneys form a pair of sophisticated and vital organs whose main function is to remove breakdown waste products of tissues found in the blood. In this way, the kidneys filter the blood and remove toxic substances such as urea, forming a dilute solution in the process, urine. The kidneys apart from being an important part of excretion and homeostasis, also have some endocrine activity.

1.1.2 Structure of the Kidney

1.1.2.1 Blood Supply

The process of the removal of harmful substances begins with the blood entering the kidney through the renal artery, diverging into afferent arterioles carrying blood into the cortex (Figure 1.1). Blood is directed from the glomerulus into the medulla, through the efferent arterioles, where they start descending into the peritubular capillaries. The peritubular capillaries form small veins which eventually merge to the large renal vein, where the blood exits the organ.

1.1.2.2 The Nephron

The cortex contains the most important part of the kidney, the nephron (Figure 1.2). Each kidney has about a million nephrons and these are the structures where the actual process of removal of metabolic waste products takes place. This functional part is composed of the glomerulus (network of blood vessels) and the tubules (urine-collecting tubes).

The filtering of the blood takes place in the glomerulus, a tight network of capillaries engulfed by podocytes. Those epithelial cells are responsible for separating the urine passing from the glomerulus ensuring only blood cells and large proteins remain in the blood. At the end of each glomerulus a tubule is found.

The renal tubules are a series of tubes that recover all the necessary nutrients, initially filtered into the urine, to the blood (proximal convoluted tubules) or carry urine from

the glomerular capsule, and through the collecting duct into the renal pelvis (distal convoluted tubules). Consequently, the urine leaves the kidney through the ureter and into the bladder.

Figure 1.1 Cross section of a kidney

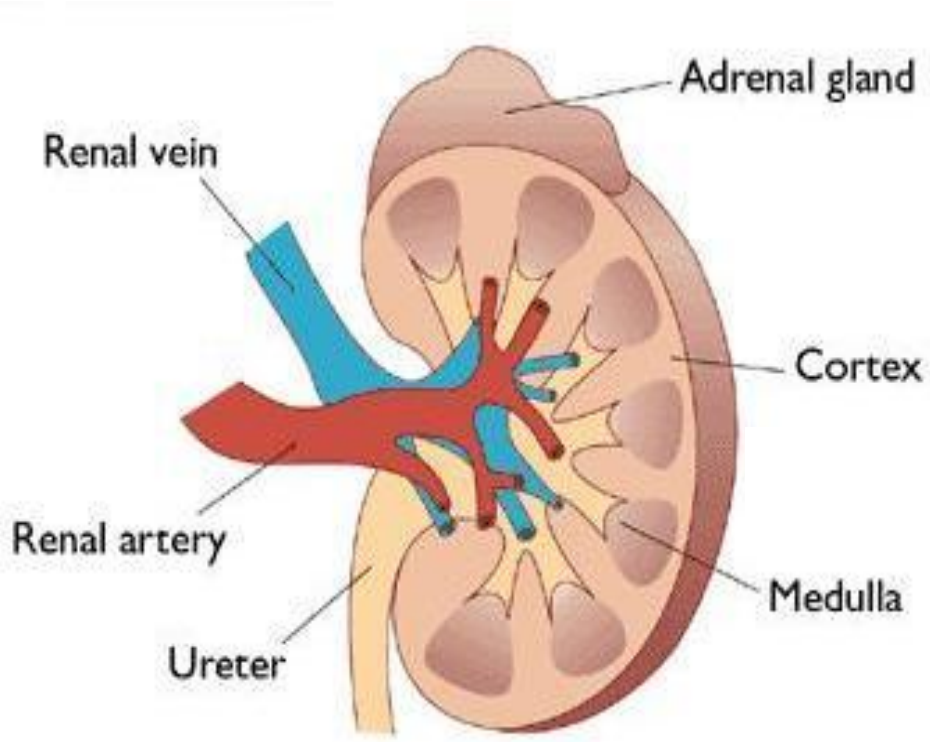
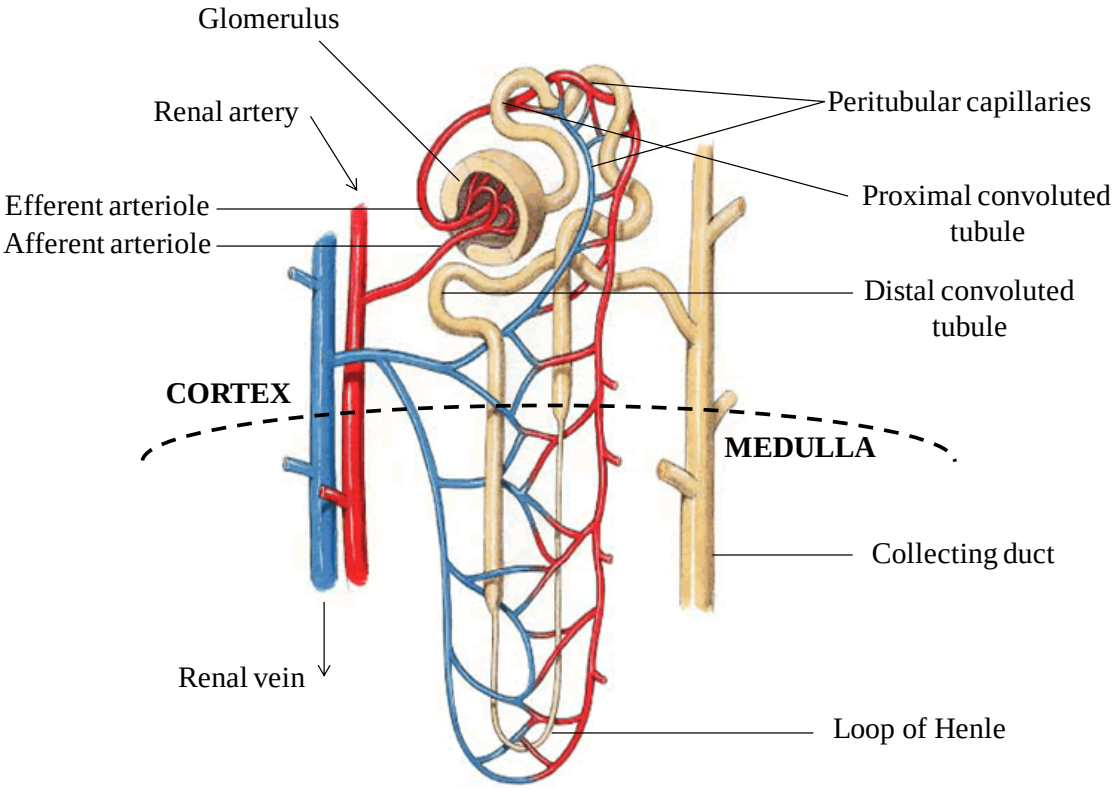


Figure 1.2 The nephron



1.2 Transplantation

The concept of organ transplantation for therapeutic purposes dates back to ancient times and in particular Hua-To (136-208 A.D.) who replaced failing organs with healthy ones, in patients who were anaesthetized with a mixture containing Indian hemp. The realization that organ rejection was an immunological response came as early as the 16th century. Italian surgeon Gasparo Taggliacozzi, known for his method of reconstructing noses and ears from patients' skin, noticed that skin from a different donor often led to the failure of the procedure. This would be recognized later, by his successors, as transplant rejection.

The era of modern transplantation began at the beginning of the 20th century. In 1902, in Vienna, Emerich Ullmann conducted kidney transplantation experiments in dogs making him the first surgeon to perform allotransplantation experiments (Druml, 2002 **1**). In 1906, Mathieu Jaboulay performed the first transplants, between species, by connecting the renal vessels of a sheep and a pig kidney to the brachial vessels in two patients with renal failure (Morris PJ, 2004 **2**). Subsequent attempts failed because of vascular thrombosis. Against a background of failure, the first successful transplantation took place in 1954, when a team of doctors in Boston, transplanted a kidney from a healthy individual to his twin brother. The graft was accepted, since donor and recipient were genetically identical (Merrill et al., 1956 **3**). However, it was not until Peter Medawar's groundbreaking skin transplantation studies in animal models, that a better understanding was obtained and led to its clarification as a form of specific immunity (Medawar PB, 1944 **4**; Medawar PB, 1945 **5**). With this came the realization that immunosuppression was required for successful transplantation and marked the beginning of the use of anti-rejection drugs. During the 60's and 70's, progress was slow with total-body irradiation achieving immunosuppression but being lethal for the majority of patients (Murray JE et al., 1960 **6**; Murray JE et al., 1962 **7**) and the use of 6-mercaptopurine which was later replaced by the less toxic azathioprine (AZA). This period was characterized by high mortality among patients until cyclosporine A (CsA) was introduced to the clinic in the early 1980's. The use of the cyclosporine A calcineurin inhibitor dramatically improved graft outcome. Currently, a variety of immunosuppressive drugs are available for use in the transplant clinic including tacrolimus (FK-506), mycophenolate mofetil (MMF) and rapamycin and so on, each with different specificity, efficacy and safety.

1.3 Effector Mechanisms in Allograft Rejection

In the absence of immunosuppression, the transplantation of tissue from one individual to another of the same species (allotransplantation) results in rejection of non-self by the recipient. Foreign antigens, provided by the allograft, are responsible for the activation of the recipient's immune response which ultimately results in injury to and destruction of the transplanted organ. There are three major immune elements which contribute to graft rejection by disparate mechanisms, the contribution of which may vary and all of which may act simultaneously (Rocha PN et al., 2003 **8**):

1. $CD4^+$ T-helper cells directed against donor antigens, activate monocytes and macrophages by secretion of proinflammatory cytokines and chemokines by a delayed type hypersensitivity response (DTH).

2. Alloreactive $CD8^+$ cytotoxic T-cells directly kill graft endothelial and parenchymal cells.

3. Donor specific antibody (DSA) binds to the graft endothelium, activates complement and coagulation, resulting in tissue injury and rejection.

Graft rejection is characterized as hyperacute, acute or chronic; a classification established according to the time course of rejection and not the mechanisms (i.e cellular or humoral) involved (Colvin RB et al., 2005 **9**). Another category of rejection which has recently been described, is subclinical rejection, which manifests itself as the rare occurrence of acute cellular rejection in the absence of graft dysfunction (Böhmig GA et al., 2004 **10**). Indeed there can be resistance of a transplanted organ to injury, in the presence of anti-donor antibodies against donor endothelial cells, a situation known as accommodation (Dehoux JP et al., 2008 **11**). Most importantly, the histopathologic pattern is what determines the type of graft rejection. The different types of rejection and their characteristics are described in table 1.1. The main subject of this thesis is humoral-mediated rejection.

*Table 1.1 Types and characteristics of renal allograft rejection (adapted from Colvin RB, 2007 **12**)*

	Clinical presentation	**Histology**	**Pathology Complement deposition (C4d)**	**Serology**	**Outcome**
Hyperacute rejection	Immediate graft loss (minutes to hours post reperfusion)	Neutrophils in glomeruli and peritubular capillaries (PTC); microvascular thombosis, haemorrhage, fibrinoid necrosis	Positive PTC; early biopsies may be negative	Anti- HLA (class I and/or II) or ABO antibody titres	Irreversible
Acute humoral rejection	Rapid (within days) clinical deterioration of graft (can occur any time post transplantation)	Neutrophils and macrophages in glomeruli and PTC; microvascular thombosis, fibrinoid necrosis, acute tubular infarction; ±cell-mediated rejection	Positive PTC; variable glomeruli	Anti- HLA (class I and/or II) or ABO antibody titres	Almost always reversible with appropriate treatment
Acute cellular rejection	Rapid (within days) clinical deterioration of graft (can occur any time post transplantation)	Accumulation of mononuclear cells in the interstitium and tubules (tubulitis); subendothelial infiltrates of $CD4^+$ and $CD8^+$ T-cells and macrophages (arteritis)	Negative unless ongoing with acute humoral rejection	Often accompanied by anti-graft antibodies	Reversible with appropriate treatment

Chronic humoral rejection	Slow, progressive (within months to years) clinical deterioration of graft function	Glomerular basement membrane (GBM) duplication; mononuclear cells in glomeruli and PTC; intimal/interstitial fibrosis, tubular atrophy; PTC basement membrane multilamination	Positive; very often negative PTC; glomeruli irregularly positive	Anti- HLA (specially class II) antibody; variable; often non-specific/ negative	Challenging. Therapy not properly defined yet
Chronic cellular rejection	Slow, progressive (within months to years) clinical deterioration of graft function	Arterial intimal fibrosis with presence of macrophages and $CD3^+$ cells within the thickened intima (chronic allograft arteriopathy); formation of neointima	Absence or presence of C4d	Often accompanied with anti-graft antibodies	Challenging. Therapy not properly defined yet
Subclinical rejection	Normal graft function	Tubulo-interstitial infiltrates (increased expression of $CD8^+$ T-cells)	Absence or presence of C4d	Often accompanied with anti-graft antibodies	Reversible with appropriate treatment. Often a prediction for chronic damage and deterioration of graft
Accommodation	Normal graft function	Normal, or minor changes	Positive PTC; variable glomeruli	Common with ABO antibodies; not well documented with HLA antibodies	Graft becomes tolerant. Mechanisms not yet defined

1.4 The Role of B cells in Clinical Transplantation

Solid-organ transplantation is the preferred treatment for patients with end-stage kidney disease (Wolfe RA et al., 1999 **13**). For decades, T lymphocytes seemed to be the predominant cells targeted in preventing kidney rejection. As a result, they became the main focus of experimental transplantation and less research was focused on alloantibody and B lymphocytes. As the barrier of T-cell-mediated rejection was gradually overcome, by the plethora of T-cell targeted therapies, interest in humoral responses re-emerged. It was only then that it became evident that DSA was not only responsible for hyperacute rejection, but was also strongly associated with an increased incidence of acute rejection incidents as well as chronic damage to the kidney (Stegall MD et al., 2010 **14**). This realization prompted transplant researchers to achieve a better understanding of the genesis of the alloantibody response, a response which involves a variety of cells with different functions and characteristics (Clatworthy MR et al., 2011 **15**).

B cells are a cell population of lymphoid origin, which overall express a variety of cell surface immunoglobulin (Ig) receptors. The B cell population as a whole recognizes multiple specific antigenic epitopes, thus making the B cell impressively diverse (Parsons RF et al., 2012 **16**). Importantly, throughout their development and maturation, different surface markers are expressed, such as CD19, CD20, CD22, CD23, CD27 and CD38 which often reflect different functions (Clatworthy MR, 2013 **17**).

Traditionally, there are two B cell subsets, which can be distinguished based on their requirement for T-cell help for activation, expansion and differentiation (LeBien TW et al., 2008 **18**). Bone marrow derived B1 cells, generated during foetal development, are T-cell independent (T-I) and reside within pleural/peritoneal cavities and in few numbers within the spleen (Clatworthy MR, 2013 **17**). They mainly produce low affinity IgM antibodies specific for multimeric sugar and lipid (T-I) antigens (Yammani RD et al., 2013 **19**). B2 cells, often considered the conventional B cells, are T-cell dependent (T-D) and bone marrow derived. They are responsible for the development of high affinity antibodies and long-lasting specific immunological memory (Tangye SG, 2013 **20**). A rare, more recently described subset of B cells, are B10 cells. These participate in the negative regulation of inflammation rather than activation of immune responses (DiLillo DJ et al., 2010 **21**). Their inhibitory activity is mediated by their

ability to express IL-10, thus often referred to as B10 cells, and constitute 1%-3% of B cells residing in the spleen (Iwata Y et al., 2011 **22**).

B cell activation is a complicated, multistep process which occurs upon encounter with an antigen in a secondary lymphoid organ (spleen/lymph node) (Parsons RF et al., 2011 **16**). Once activated, B cells enter the T cell areas generating either plasmablasts (early short lived plasma cells) which secrete low affinity antibody or they enter the germinal centre reaction (GC) (Stegall MD et al., 2010 **14**). In this environment, B cells undergo clonal expansion, somatic hypermutation (SHM) and isotype class switch recombination (CSR). These cells eventually differentiate into memory B cells and long lived antibody secreting cells (ASCs) (Clatworthy MR et al., 2010 **23**).

In principle, a strong alloantibody response requires activation of T-D B cells. During the sensitisation period, T cells are presented with the alloantigen through the direct pathway in the grafts and at a later stage, through the indirect pathway in the lymph nodes (Baratin M et al., 2004 **24**). Antigen specific B cells internalize epitopes from allogeneic cells, and through their surface major histocompatibility complex (MHC) II molecules, these are presented to $CD4^+$ T-helper cells (Mitchison MA et al., 2004 **25**). Studies in mice have demonstrated that for a proper IgG-mediated response the above pathway is required (Steele DJ et al., 1996 **26**). From that point onwards, long-lived plasma cells become permanently differentiated and migrate to the bone marrow synthesizing antibody indefinitely, in the absence of T cells (Shapiro-Shelef M et al., 2005 **27**). On the other hand memory B cells are generated, and although they do not secrete antibody, upon re-stimulation with alloantigen they rapidly transformed to ASCs (Yoshida T et al., 2010 **28**).

1.5 Hyperacute Rejection (HAR)

HAR is the most exuberant example of antibody-mediated rejection (AMR). It affects patients who are pre-sensitised either due to antibodies against ABO blood group antigens or HLA antigens. Preformed antibodies (DSA) in the blood of the recipient pose a significant barrier to transplantation. Those antibodies against the donor organ are responsible for immediate graft loss, which can take place within minutes post-revascularization (Singh N et al., 2009 **29**). This instant and complex response involves the binding of these antibodies to a variety of antigens expressed on the surface of the donor endothelium, triggering the activation of the complement and coagulation

cascades which lead to graft injury. In renal transplantation, the graft lacks normal reperfusion and as a result the kidney looks flaccid, mottled and in extreme cases completely black, resulting in the patient undergoing a nephrectomy (Nankivell BJ et al., 2010 **30**). Tissue is deeply characterized by major neutrophilic infiltration, extended coagulopathy and overall infarction (Table 1.1).

1.5.1 ABO Antigens

In the early days of transplantation, HAR often resulted from high titres of pre-existing IgM antibody directed against a foreign antigen. Such "natural antibodies" were generated in response to carbohydrate epitopes on glycolipids and glycoproteins which pre-existed in a naive host prior to transfusion. The classical example of these are antibodies against the ABO blood group antigens, specific for A and B antigens (Colvin RB et al., 2005 **9**). These oligosaccharide antigens are generated by glycosyltransferases, present in the Golgi apparatus responsible for the biosynthesis of polysaccharides on lipids and proteins (Shaper JH et al., 1992 **31**). They are not only found on the surface of red blood cells, white cells and platelets but are also widely distributed on endothelial cells and renal tubular basement membranes (Yaich S, 2013 **32**). Successful transplantation across an ABO barrier has been achieved using immunoadsorption and plasmapheresis protocols which non-selectively remove all antibodies from the circulation (Genberg H et al., 2010 **33**). Following these procedures, anti-ABO antibodies return, often at pre-operative levels, yet they generally do not damage the endothelium. This unusual phenomenon of the organ resisting HAR has been described as 'accommodation', a mechanism not fully understood (Chopek MW et al., 1987 **34**).

1.5.2 α1,3GT Antigens

In contrast to ABO-incompatible allotransplantation, HAR still remains the main obstacle to xenotransplantation. ABO-incompatible transplantation and xenotransplantation share many similarities, most importantly in that the xenoepitope, generated by the activity of α1,3galactosyltransferase (α1,3GT), bears a striking resemblance to the structure of blood groups A and B (Milland J et al., 2006 **35**). The presence of natural xenoreactive antibodies against the carbohydrate galactose-α-1,3-galactose (Galα1, 3Gal, α-gal) prevents transplantation of xenogeneic pig organs to human recipients (Parker W et al., 1994 **36**). Furthermore, xenografts are especially

vulnerable to damage mediated by complement, since porcine complement regulators fail to suppress human complement activation on the pig endothelium (McCurry KR et al., 1995 **37**). As a result, the development of xenotransplantation, which is being considered as a solution to the lack of human organs, still has a long way to go before being a clinical possibility. However, the resemblance between the α-gal epitope and the ABO antigens provides valuable information regarding carbohydrate biology, giving insight into the role of antibodies in HAR (Galili U, 2006 **38**).

1.5.3 HLA Antigens

In the current clinical setting, the focus is on antibodies against protein alloantigens such as the major histocompatibility antigens expressed on the surface of donor cells. Patients may be sensitised to MHC antigens by blood transfusions, pregnancies or rejection of a previous graft, leading to the recipient developing anti-human leukocyte antigen (HLA) antibodies against the donor organ tissue. Since, in this case, the recipient's immunological reactivity is generated prior to transplantation, the subsequent graft may be rejected in a hyperacute manner, usually mediated by antibodies of the IgG subtype (Steele DJ et al., 1996 **26**). The main antigenic targets being allogeneic MHC Class I and II molecules (van Saase JL et al., 1995 **39**). MHC Class I molecules are expressed on the surface of all nucleated cells including endothelial cells whereas MHC Class II molecules on resting cells, have a more restricted expression including on dendritic cells (DCs), B cells and vascular (capillaries, veins and arteries) endothelial cells (Baldwin WM III et al., 2010 **40**). In contrast to human endothelium, murine endothelium does not express class II antigens in the resting state, however upon activation due to local inflammation, MHC II expression of antigens can be rapidly induced (Goes N et al., 1995 **41**).

The HLA system is a series of genes located on chromosome 6. There are approximately 200 genes which are heavily involved in antigenic presentation to T cells and some of them display extreme polymorphism (Becks S et al, 2000 **42)**. They are categorized into 2 main groups: MHC Class I and II antigens. There are three class I MHC proteins in humans, HLA, -B and -C, and there are three class II MHC proteins HLA-DR, -DQ and –DP (Sheldon S et al., 2006 **43**). These genes are the most polymorphic loci in the human genome, with some having hundreds of alleles (Erlich HA et al., 2001 **44**). The extreme polymorphism of the HLA enables antigen presenting

cells (APCs) to display a vast repertoire of peptide fragments to T cell receptors (Nankivell BJ et al., 2010 **30**). Small discrepancies between donor and recipient in amino acid sequence, within the MHC peptide-binding groove is enough to alter peptide presentation and elicit severe graft rejection. In general, the HLA class I molecules consist of an α and β_2 microglobulin chain and present endogenous antigen to $CD8^+$ T cells. The HLA Class II molecules consist of an α and β chain and present peptide of exogenous antigens to $CD4^+$ T cells (Neefjes J et al., 2011 **45**).

HAR no longer poses a threat to clinical transplantation due to crossmatching, which was introduced in 1969 (Patel R et al., 1969 **46**). This involves testing the recipients' serum for the presence of preformed antibodies against the potential donor. This has significantly reduced the risk of HAR. Methods for detection have been improved overtime and currently include highly sensitive solid-phase immunoassays (SPI) (Mahdi BM, 2013 **47**).

1.5.4 MHC Antigens in the Rat

Histocompatibility research has led to dissection of the rodent MHC. In the rat, the RT1 complex is the major histocompatibility complex in and like the MHC of other species, it comprises a group of closely related genes (Günther E et al., 2001 **48**) and is located on the RNO chromosome 20 (Locker J et al., 1990 **49**). The rat has been consistently used in transplantation studies and the antigen-specific immune responses have been attributed to class I genes, RT1.A, RT1.C and RT1. E and class II genes, which are found in the RT1B/D region (Palmer M et al., 1983 **50**). Strong graft rejection has been closely associated to RT1.A and RT1.B/D (Günther E et al., 2001 **48**; Günther E et al., 1984 **51**). The RT1.A genes are analogous to the mouse H-2K, D and L (Günther E et al., 2001 **48**) and exhibit a similar level of polymorphism to the mouse H-2 (Cami B et al., 1981 **52**; Pease LR et al., 1982 **53**).

1.6 Acute Antibody-Mediated Rejection

Although the utilization of sensitive crossmatching techniques has virtually eliminated HAR, antibodies play a significant role in acute allograft dysfunction post transplantation. First of all, presensitisation still remains a major risk factor as it poses a serious threat for an anamnestic alloantibody response. This memory immune response is generated by past exposure to the donor specific antigen, generating high titres of

complement-fixing antibody upon re-exposure (Terasaki PI, 2003 **54**). Secondly, although patients can have a negative crossmatch, they may develop a primary immune response with *de novo* synthesis of DSA. AAMR manifests itself as a sudden deterioration of graft function which usually takes place within days or a few weeks after transplantation (see table 1.1). Nonetheless, AAMR can occur years after transplantation, often due to changes in the recipient's immunosuppression regimen (Colvin RB, 2007 **12**). As with HAR, the main targets of preformed and *de novo* antibodies are the MHC and ABO antigens on the donor endothelium, however, all antigens expressed on endothelial cells (ECs) can potentially serve as targets. Examples include minor histocompatibility antigens (miHA) and non-HLA antigens. Upon histopathological analysis, the injury mediated by these antibodies displays similar morphologic characteristics to HAR, although as a general rule AAMR is less severe (Puttarajappa C et al., 2012 **55**). At present, AAMR is well characterized in the human renal biopsies (Table 1.2) (Truong LD et al., 2007 **56**). A major contribution to the diagnosis of AAMR has been made by the introduction of staining for the deposition of complement fragment, C4d, a marker of classical pathway complement activation, into routine transplant pathology. This was initially proposed by Feucht (Feucht HE et al., 1993 **57**) and it reveals a strong correlation between DSA and complement activation.

1.7 Chronic Antibody-Mediated Rejection

There is strong recent evidence supporting the involvement of HLA antibodies in the slow deterioration of graft function. Dysfunction is manifested in structural changes in the microcirculation (Table 1.1) which involves glomeruli and PTC, arteries and general damage of the kidney through interstitial fibrosis and tubular atrophy (Mengel M et al., 2012 **58**). This type of rejection is termed chronic antibody-mediated rejection (CAMR) and is considered to be a major complication and challenge in the long-term survival of kidney grafts, despite the plethora of immunosuppressive agents and sensitive HLA screening assays (Carter V et al., 2005 **59**). Due to its arduous nature, multiple controversies have arisen regarding CAMR. First of all, there is a debate concerning the definition of "chronic" rejection; is it simply an established scar formation or active chronic changes which therefore have the potential for treatment (Cohen D et al., 2011 **60**)? Regarding the second hypothesis, DSA to HLA antigens can promote rejection with subsequent complement activation, thus making C4d a suitable marker of an

ongoing immunological reaction (Colvin RB, 2007 **12**). DSA, especially specific for allogeneic MHC class II, has been associated with transplant glomerulopathy and arteriopathy and there are many studies highlighting the strong association of those histological features with C4d (Regele H et al., 2002 **61**; Rotman S et al., 2005 **62**; Lorenz M et al., 2004 **63**). In addition, studies have revealed that patients positive for C4d, had greater chances of developing CAMR, leading to the assumption that this biomarker predicts early stage of glomerulopathy (Regele H et al., 2002 **61**; Loupy A et al., 2009 **64**). Secondly, although a combination of alloantibody and C4d deposition has been an acceptable marker for the diagnosis of CAMR, there is a substantial number of patients exhibiting all the histological features of chronic graft impairment but in the absence of HLA antibody (Akalin E et al., 2007 **65)** or C4d staining at the time of biopsy. Studies where there is a lack of C4d as a marker (Al Aly Z et al 2005 **66**; Sis B et al., 2007 **67**) propose a form of humoral rejection (in a chronic but not acute background) whose mechanism is complement independent.

Antibodies, regardless of whether they fix complement or not, have the ability to alter the activation state of the donor endothelium upon binding the cells. Consequently, they can induce proinflammatory responses which recruit cells of the innate arm of the immune system (Lee CY et al 2005 **68**) leading to scarring of the graft. Adding to the challenge of CAMR is the well established fact that chronic allograft rejection can take place in the absence of any HLA-specific antibody (Rose ML, 2004 **69**). Furthermore, autoimmune responses may contribute to graft loss through exposure of peripheral self-antigens, adding to existing damage caused by alloantibody. These non-HLA- specific auto-antibodies are directed against targets such vimentin (Carter V et al., 2005 **59**) which is not normally expressed on the endothelium but maybe induced upon inflammation or angiotensin II type 1 (AT_1) receptor (Dragun D, 2007 **70**). In addition, glutathione-S transferase (GSTT1) expression can promote damage to the graft, when the organ from a GSTT1-positive donor is given to a GSTT1-negative recipient (Akgul SU et al., 2013 **71**). Finally, polymorphic endothelial MHC-class I related chain A (MICA) and chain B (MICB) antigens participate in chronic rejection (as well as HAR and AAMR). These host-specific antigens, including miHA, are of particular interest since they escape detection by traditional cross-matching techniques (Grafft CA et al., 2010 **72**). Increasing evidence that highlights the impact of non-HLA-specific antibodies to graft loss is accumulating. Recent data reveal that 10-23% of renal

transplant recipients are presensitised to non-HLA antigens (Jackson AM et al., 2011 **73**; Qin Z et al., 2011 **74**) and 22% develop *de novo* non-HLA-specific antibodies after transplantation (Sigdel TK et al., 2012 **75**). For this reason, high-density arrays are being used in research to identify target antigens for non-HLA-specific anti-endothelial cell antibodies (AECAs) in transplant recipients experiencing AMR (Jackson AM et al., 2014 **76**). These observations have highlighted the need for markers that use endothelial transcripts as indicators of CAMR (Sis B et al., 2010 **77**).

CAMR is a problematic condition that greatly affects graft survival. It is not known what drives antibodies to cause acute rejection in one patient and chronic in another. It is possible that factors such as antibody titre, avidity and effector mechanisms directed at the endothelium, contribute to the outcome. As a result of the extremely complex situation, a standard therapy for CAMR is currently not agreed (Cohen D et al., 2012 **60**).

*Table 1.2. Diagnostic criteria for acute antibody-mediated rejection (adapted from Haas M et al., 2014 **78**)*

1. Morphologic evidence of acute tissue injury
    I. Acute tubular infarction
    II. Neutrophils and/or mononuclear cells in peritubular and/or glomerular capillary
    III. Glomeruli and/or capillary thrombosis; arterial fibrinoid necrosis
2. Immunopathological evidence for the action of antibodies
    I. C4d deposition in PTC and GBM (less often)
    II. Presence of immunoglobulin and complement in PTC and arteries (rare)
3. Serological evidence of HLA or other anti-donor endothelial antibodies

1.8 Effector Mechanisms of Antibody-Mediated Rejection

1.8.1 Resting vs Activated Endothelium

Microvascular endothelium is the primary interface between the transplanted organ and the recipient's immune response (Turgeon NA et al., 2009 **79**). Resting endothelium is a well-organized network of tubules with distinct morphology which strongly supports a monolayer of endothelial cells connected at tight junctions. The undisturbed endothelium, free of any surface disruptive stimuli, is crucial to providing a barrier between the vascular and extra vascular spaces (Liptak P et al., 2005 **80**). The main target of antibody-mediated rejection is the glomerular and PTC endothelial compartment of the kidney. Here, high levels of DSA against both MHC I and II antigens directly activate the endothelium promoting cell lysis and death. In addition, the anti-HLA antibodies mediate complement activation and rapid upregulation of adhesion molecules and growth factor expression (Table 1.3) (Wehner J et al., 2007 **81**). These lead to inflammation of the microvasculature and breakdown of the endothelial barrier with leakage of intravascular fluids to the tissue. In addition, there is platelet aggregation/coagulation cascade leading to cell apoptosis and ultimately destruction and rejection of the graft (Liptak P et al., 2005 **80**; Dranchenberg CB et al., 2013 **82**).

Table 1.3. Chemokines, adhesion molecules and growth factors expressed by endothelial cells in response to both antibody and complement (adapted from Wehner J et al., 2007 ***81****)*

Stimulants	Mediators	Function
Anti-MHC antibody; C5a, MAC	von Willebrand factor; P-selectin; VCAM-1, ICAM-1	Adhesion
Anti-MHC antibody; C1q, C3a, C5a, MAC	MCP-1, IL-8, CCL5	Chemotaxis, Transmigration
Anti-MHC antibody; MAC	PDGF, VEGF, basic FGF	Growth factors

1.8.2 Direct Effects of DSA on the Donor Endothelium

1.8.2.1 Crosslinking of MHC antigens by Antibodies results in Endothelial and Smooth Muscle Cell Proliferation

Exposure of alloantibodies to human HLA antigens displayed on the graft has multiple effects on the donor endothelial cells. *In vitro* experiments have revealed that cross linking of the antigens by antibodies induces complement-independent upregulation of surface proteins as well as intracellular signalling by growth factor receptors (Naemi FM et al., 2013 **83**). The consequent induction of integrins and growth factors, such as basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) is associated with cell proliferation and survival (Zhang X et al., 2012 **84**). Interestingly, the higher the concentration of anti-HLA Class I antibody the stronger the stimulus for FGF receptor expression on the EC surface (Jindra PT et al., 2006 **85**). In addition, expression of another growth factor, PDGF (platelet-derived growth factor) serves as a potent mitogen for cells of mesenchymal origin (Bunte MC et al., 2008 **86**). Finally, engagement of alloantibody to their antigenic targets activates transcription factors, such as nuclear factor-kB (NF-κB). These are responsible for proinflammatory and procoagulant cell mediated proliferation (Zhang X et al., 2009 **87**).

1.8.2.2 Crosslinking of MHC antigens by Antibodies activates Leukocytes through Fc Receptors

Accumulation of leukocytes on injured vessel walls can be mediated by signalling through their Fc Receptors (FcRs). Binding of these receptors to the Fc portion of the tissue ligated antibody, activates the leukocyte to strongly promote graft injury (Joudeh A et al., 2013 **88**). Furthermore, it provides an important link between the innate and adaptive immune responses (Nimmerjahn F et al., 2007 **89**). There are four main types of Fc receptors: FcγRI, FcγRIII, FcγRIV and inhibitory FcγRIIB expressed by a variety of inflammatory cells such as neutrophils, monocytes/macrophages, and NK cells (Sunay Erdinc MM et al., 2013 **90**). The responses of these Fc-bearing inflammatory cells to bound antibodies, cause damage to the transplanted tissue. This can be the result of cytokine and chemokine release, leukocyte degranulation leading to phagocytosis and apoptosis through antibody-dependent cellular cytotoxicity (ADCC) (Joudeh A et al., 2013 **88**). These mechanisms that potentiate graft rejection heavily

depend on the specificity of the FcRs involved. Neutrophils and macrophages express high affinity FcγRIII (CD16) (Giorgini A et al., 2008 **91**) and FcγRI (CD64) (Nimmerjahn F et al., 2008 **92**) respectively, and low affinity FcγRIV and inhibitory FcγRIIB (CD32) (Baldwin WM 3rd et al., 2012 **93**). Activation of these cells results in upregulation of proinflammatory MCP-1, IL-8, IL-6 (Wasowska BA, 2010 **94**) and induction of TNF-α and IL-1-α (Abrahams VM et al., 2000 **95**). Other cells expressing FcγRIIB include endothelial cells and platelets although their function is not well understood (Wehner J et al., 2007 **81**). Interestingly, B cells which express inhibitory FcγRIIB, and NK cells, are the only cell types that can express only activating or inhibitory receptors alone (Nimmerjahn F et al., 2007 **89**). NK cells mediate ADCC through the release of cytotoxic granules containing perforins and granzymes, at the site of injury, leading to cell apoptosis and graft rejection (Lee CY et al., 2007 **47**). In addition, NK cells, through activation of FcγRIII can mediate elevated expression of multiple cytokines such as IFN-γ, a major participant in allograft rejection (Akiyoshi T et al., 2012 **96**).

1.8.2.3 Crosslinking of MHC antigens by Antibodies promotes Leukocyte Adhesion and Chemotaxis

As previously stated, antibody ligation on the graft's surface induces growth factors, adhesion molecule expression and cytokine production (Zhang X et al., 2009 **87**). Studies on acutely rejected renal allografts showed upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelium (Lucchiari N et al., 2000 **97**). Interaction of adhesion molecules with surface receptors on leukocytes results in the migration of cells through the basement membrane (Yadav R et al., 2003 **98**). Furthermore, recent data have indicated that bivalent IgG interactions with allo MHC antigens, stimulates rapid exocytosis of von Willebrand factor (vWf) from the subenthothelium. vWf is an important link between endothelial cell activation, platelet aggregation and leukocyte recruitment (Morrell CN et al., 2007 **99**). Upon release of vWf, a series of events is initiated which is not only proinflammatory but procoagulant as well. This protein is the initial stimulus for platelet adherence to the endothelium and consequently aggregation of activated platelets (Lowenstain CJ et al., 2005 **100**). Platelets contain a plethora of molecules, such as chemokines and cytokines which strongly contribute to EC activation and leukocyte

localisation to the graft (Turgeon NA et al., 2009 **79**). Importantly, stimulated endothelial cells and activated platelets release P-selectin. P-selectin is an adhesion molecule and once externalized induces further platelet recruitment and initiates first stages of leukocyte trafficking (Celi A et al 1994 **101**). As a result, a large influx of proinflammatory cytokines, such as IL-1, IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are secreted at the site of leukocyte activation (Le Meur Y et al., 2002 **102**) further adding to tissue damage. It is clear from published data that without adhesion molecules, platelet-derived factors and inflammatory components, generated during an alloantibody response, leukocyte trafficking could not be sustained.

1.8.2.4 Crosslinking of MHC antigens by Antibodies induces Procoagulant Responses

Critically, the previously discussed alloantibody-endothelium-vWf-platelet interactions are not only associated with vascular inflammation but also create a procoagulant interface which strongly promotes thrombosis (Sis B, 2012 **103**). Activated platelets promote the release of tissue factor (TF) from the subenthothelium (Kirk AD et al., 2009 **104**). TF is an integral membrane protein and is the principal initiator of the extrinsic pathway of the blood coagulation cascade (Chu AJ, 2005 **105**). A series of *in vitro* experiments addressing xenogeneic responses, provided evidence for human stimulated platelets also releasing substantial amounts of TF on porcine endothelial cells (Bustos M et al., 2001 **106**). On the other hand, P-selectin, apart from orchestrating leukocyte reactions at the site of inflammation, is responsible for the expression of TF on the surface of polymorphonuclear (PMNs) cells and monocytes (Celi A et al 1994 **101**). As a result, TF is responsible for the initiation of the clotting cascade generating large amounts of thrombin, a serine protease which is a key potent effector of coagulation (Chen D et al., 2009 **107**). Therefore, alloantibodies against the transplanted graft may cause sufficient endothelial exocytosis of TF which in turn induces rejection by inflammation and thrombosis. Increasing evidence for the involvement of coagulation in the process of AAMR, includes functional analysis of endothelial cell-associated transcripts (ENDTAs). Interestingly, vWf was identified as the most prevalent transcript in AAMR biopsies (Sis B, 2012 **103**). *In vitro* experiments on a human endothelial cell line, stimulated with anti-MHC I and II antibodies, revealed the

speed at which vWf is released by the endothelium (within minutes) and its sustained exocytosis (60 minutes) post activation (Yamakuchi M et al., 2007 **108**).

1.9 The Blood Coagulation Cascade

1.9.1 Activation of Platelets

The surface of a quiescent endothelium provides an antithrombotic environment with a tendency for thrombosis when there is injury. With damage, the response is to promote coagulation and this is mainly controlled by the generation of thrombin (Cines DB et al., 1998 **109**). During the initiation stage of the coagulation cascade, transplant-associated endothelial injury will result in the activation of platelets and inevitably the formation of a platelet plug (Bunte MC et al., 2008 **86**). There are two distinct pathways leading to the activation of platelets: these involve subendothelial exposure of vWf and collagen and secondly, thrombin generated by tissue factor (discussed later) (Furie B et al., 2008 **110**).

Platelets express receptors that allow them to be recruited from the blood flow to the site of injury. Platelet adhesion is promoted by receptor GPVI forming low affinity interactions with collagen and the glycoprotein complex (GP) Ib-V-IX promoting strong interactions with vWf (Varga-Szabo D et al., 2008 **111**). After the initial tethering, which results in platelet activation, platelets release the contents of their alpha granules containing P-selectin, ADP and thromboxane A_2 (TXA_2). These cause further platelet aggregation and activation (Denis CV et al., 2007 **112**) which enables one of the main haemostatic functions of activated platelets, that is to provide the surface for the assembly of coagulation complexes for generation of thrombin and formation of insoluble fibrin (Jackson SP, 2011 **113**).

1.9.2 Pathways to Coagulation

The coagulation cascade has been divided into two complex pathways, the intrinsic and the extrinsic pathway, and involves numerous clotting factors (Table 1.4). The sequence of coagulation events is a series of conversions of inactive zymogens to activated enzymes which generally takes place on the phospholipid surfaces of endothelial cells and platelets in the presence of Ca^{++} (Camerer E et al., 1996 **114**). Both pathways

converge at the activation of factor X (common pathway) and the ultimate generation of thrombin (Tanaka KA et al., 2009 **115**).

1.9.2.1 Contact Activation (Intrinsic) Pathway

The intrinsic pathway of activation involves blood coming into contact with negatively charged surfaces. As a result, proteins such as prekallikrein (PK), high molecular weight kininogen (HMWK) and factor XII (FXII) and XI (FXI) initiate the activation cascade (Camerer E et al., 1996 **114**). Active FXIIa cleaves PK to kallikrein which in turn generates more FXII. Through numerous enzymatic conversions (Figure 1.3) FXIIa activates FXI to FXIa and in reactions that require Ca^{++} FXIa converts FIX to FIXa. Active FIXa activates FXa. This process needs the formation of a complex which is formed on the surface of the activated cell. Here, membrane bound VIIIa, which acts as a cofactor to FIXa, and forms the tenase complex, activates FX to FXa (Renné T, 2012 **116**). All the activators of the contact pathway are intrinsic to the blood, and this has led to the logical hypothesis that this pathway is initiated *in vitro* only, and that fibrin formation *in vivo*, is usually generated, if not completely, by the extrinsic (tissue factor) pathway (Renné T, 2012 **116**).

Table 1.4 Blood coagulation factors

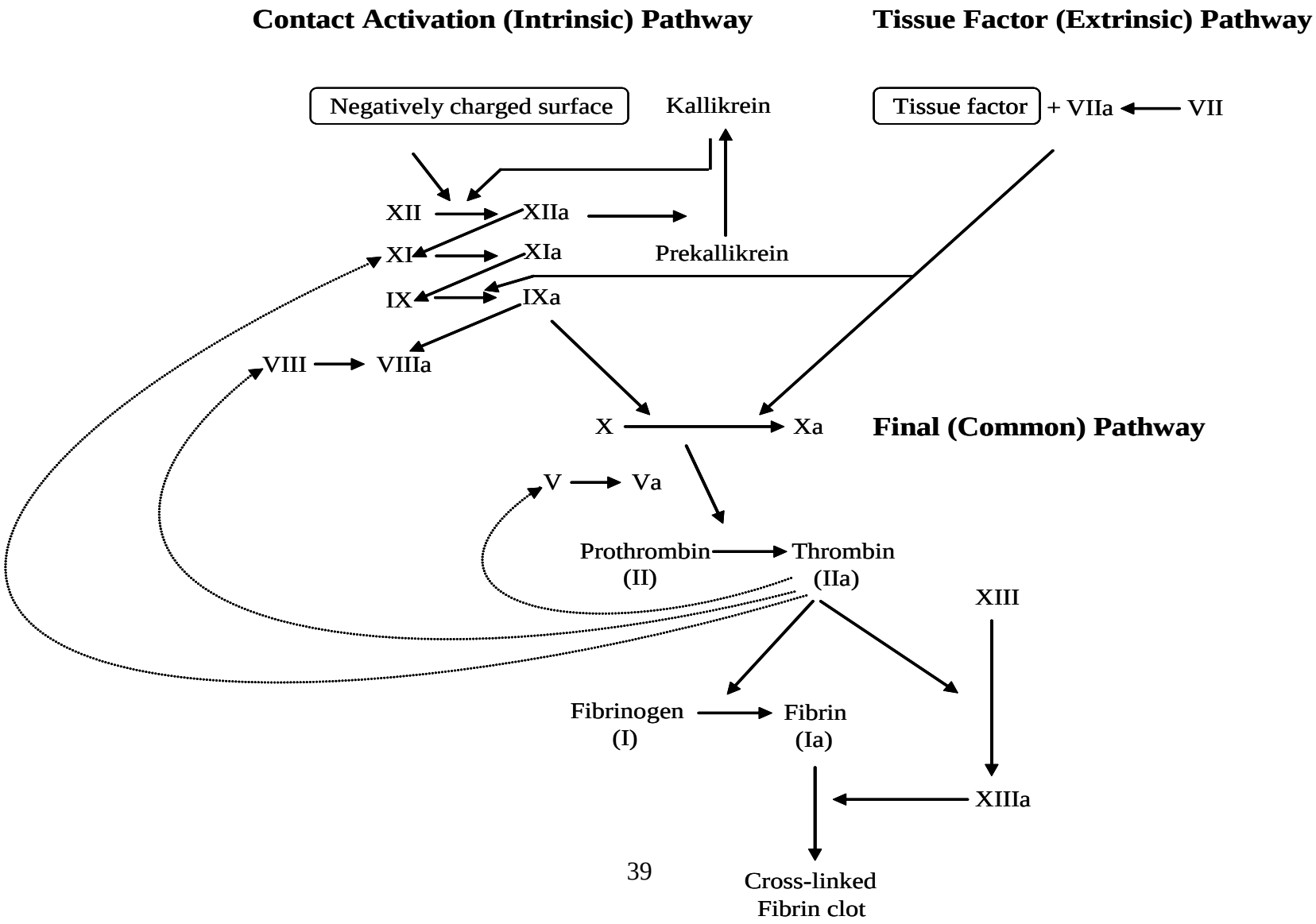
Name	Pathway	Function
High molecular weight kininogen factor (HMWK) (Fitzgerald or Flaujeac factor)	Intrinsic	Co-factor in kallikrein and XII activation
Prekallikrein (PK), Fletcher factor	Intrinsic	Activated form, kallikrein, interacts with HMWK and XII
Fibrinogen (factor I)	Both	Cleaved by thrombin to form the fibrin clot
Prothrombin (factor II)	Both	Activated form, thrombin, is the main coagulation enzyme
Tissue factor (factor III)	Extrinsic	Co-factor for factor VII, initiator of extrinsic pathway
Calcium ions (factor IV)	Both	Ca^{++} ions necessary for coagulation reactions
Factor V (Labile factor)	Both	Activates prothrombin to thrombin, forms the "prothrombinase" complex
Factor VII (Proconvertin)	Extrinsic	Forms TF-VIIa complex, initiates extrinsic pathway
Factor VIII (Antihemophilic factor)	Intrinsic	VIIIa forms the "tenase" complex, activates factor X
Factor IX (Christmas factor)	Intrinsic	IXa forms the "tenase" complex, activates factor X
Factor X (Stuart-Prower factor)	Both	Xa initiates the common pathway, forms the "prothrombinase" complex
Factor XI (plasma thromboplastin antecedent)	Intrinsic	XIa activates factor IX
Factor XII (Hageman factor)	Intrinsic	Activated form initiates the intrinsic pathway
Factor XIII (Fibrin stabilizing factor)	Both	Transamidase that stabilizes fibrin clot by cross-linking

** a refers to the activated form of the inactive precursor*

1.9.2.2 Tissue Factor (Extrinsic) Pathway

Tissue factor (TF) is the principal initiator of the extrinsic pathway at the site of injury, resulting in the sequential activation of clotting factors FVII, FX, prothrombin and the production of fibrin clot (Chu AJ, 2005 **105**). The TF pathway (Figure 1.3) can be further divided into an initiation phase and an amplification phase. The initiation phase involves TF binding circulating FVIIa to form a TF-FVIIa complex which instantly generates FXa (Thomas WS et al., 1993 **117**). Newly generated FIXa binds to its cofactor FVIIIa to form the tenase complex (in a similar fashion to the intrinsic pathway) resulting in the activation of FX. The activation of FX to FXa enters the final (common) pathway (Figure 1.3). FXa binds to membrane bound FVa, forming the prothrombinase complex witch converts prothrombin to thrombin, the final enzyme in the coagulation cascade. During this stage, small amounts of thrombin are generated which enter the amplification phase (Johari V et al., 2012 **118**). During this phase, the thrombin which has been generated, amplifies itself by activating FVIII and FV to form the tenase and prothrombinase complexes respectively. These are responsible for a second larger burst of thrombin generation (Furie B et al., 2008 **110**). Additionally, this protease has been considered to be able to activate FXI of the intrinsic pathway. This demonstrates how the components of the intrinsic pathway complement TF-mediated blood coagulation (Chu AJ, 2011 **119**). On the activated platelet surface, thrombin concentrates fibrinogen which is converted to fibrin, a requirement for a stable haemostatic plug (Ni H et al., 2000 **120**). Finally, thrombin promotes polymerization of fibrin monomers, through activation of plasma and platelet FXIII (Tanaka KA et al., 2009 **115**), resulting in a cross-linked fibrin clot.

Figure 1.3 The blood coagulation cascade



1.9.3 Key Mediators of Coagulation

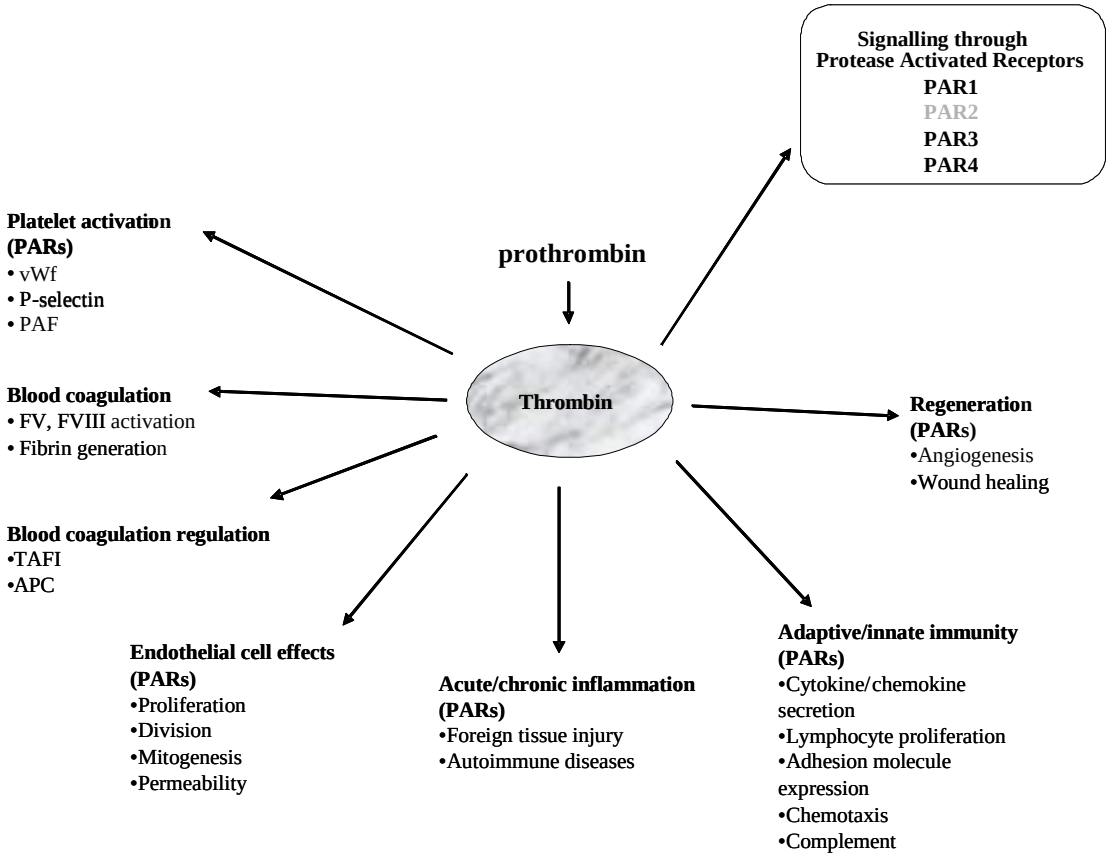
The coagulation cascade is a tightly regulated network of catalytic reactions undertaken by active coagulation components. However, these mediators promote responses which extend further than thrombosis. They are major initiators of inflammation. Among these, is TF which mediates increased vascular permeability through the up regulation of IL-1β (Puhlmann M et al., 2005 **121**), and plasma levels of IL-6 (Chu AJ, 2011 **119**). Absence of coagulation factor VIIa impairs inflammatory responses (Xu H., 2006 **122**) whereas FXa correlates with expression of IL-8, monocyte chemotactic protein-1 (MCP-1), and the upregulation of adhesion molecule expression (Senden NH., 1998 **123**). Nonetheless, it is the serine protease thrombin which is the central modulator of haemostasis and vascular inflammation (Fenton II JW et al., 1991 **124**) and this will be discussed later.

1.9.3.1 Thrombin

Thrombin is a mulfunction serine protease (Figure 1.4) generated at sites of tissue injury, thus making it a key regulator of homeostatic processes, and the main effector enzyme in the blood coagulation system (Coughlin SR, 2005 **125**). Its role in inflammation is well characterized and in general, these type of responses can be amplified through thrombosis (generation of fibrin) or signalling through protease activated receptors (PARs) (discussed later) (Chen D et al., 2009 **126)**.

As previously stated, TF is the principle initiator of the extrinsic pathway of coagulation and therefore important to thrombin generation. Upon activation, a series of positive feedback loops amplify further thrombin resulting in extensive cross-linked fibrin clots (Figure 1.3). Fibrin, apart from being important in stabilizing the haemostatic plug, is in fact highly proinflammatory. It induces NF-κB (Liu X et al., 2000 **127**), and mediates the release of IL-1β and IL-8 (Lee ME et al., 2001 **128**; Liu X et al., 2000 **127**). In addition to thrombin's amplification role, it is also capable of negatively regulating thrombotic responses by activating protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) (Tanaka KA et al., 2009 **115**).

Figure 1.4 Functions of thrombin



Thrombin can have direct effects on a variety of cells, such as platelets, smooth muscle cells and endothelial cells (Coughlin SR, 2000 **129**). These cellular responses to thrombin are mediated by a family of G protein-coupled protease-activated receptors (PARs) (Coughlin SR, 2005 **125**). These receptors are unique in the aspect that they carry their own ligand which remains masked unless the receptors are cleaved to reveal the tethered ligand (Danckwardt S et al., 2013 **130**). Thrombin is responsible for the proteolytic cleavage and activation of the receptor which consequently induces autocrine and paracrine signalling (Chen D et al., 2009 **126)**.

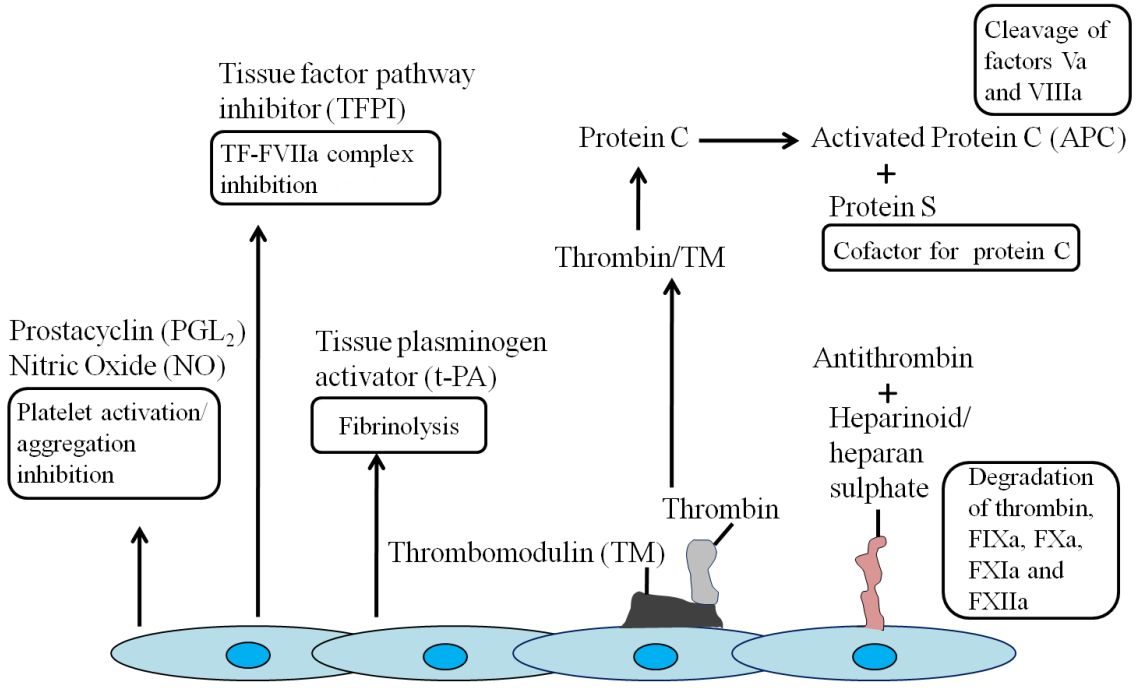
Thrombin is without doubt, the most potent platelet agonist. Platelet activation, which is crucial for effective haemostasis, is mediated by surface expression of PAR1 and PAR2. Thrombin interaction with either receptor, leads to the activation and further aggregation of platelets (Coughlin SR, 2005 **125**) although it has been suggested that the role of PAR1 might be most important (Covic L et al., 2000 **131**). In contrast to

human platelets, mouse platelets utilise PAR3 and PAR4 to respond to thrombin (Kahn ML et al., 1998 **132**). Activation results in the expression of a plethora of immune-modulatory molecules, such as adhesion molecules, chemokines and coagulation factors (Popović M et al., 2012 **133**). Furthermore, the interaction of platelets with thrombin leads to the production of microparticles (MPs) which are generated by budding of the cell membrane upon disruption of the phospholipid bilayer (Herring JM et al., 2013 **134**). MPs are rich in TF and contain surface receptors for FVIII and FV (Barry OP et al., 1999 **135**) providing procoagulant activity close to the platelet activation site. In addition, platelet derived MPs contain P-selectin glycoprotein ligand-1 (PSGL-1) which promotes platelet-platelet interactions and aggregation (Fourcade O et al., 1995 **136**). Finally, thrombin can activate, through PARs, vesiculation of both leukocytes and endothelial cells. Leukocyte-derived MPs bearing TF, increase its concentration at the inflamed site (Popović M et al., 2012 **133**) and they stimulate inflammatory responses through expression of cell adhesion molecules (Herring JM et al., 2013 **134**). Finally, vesicle shedding from endothelial cells stimulated by thrombin, results in increased endothelial-leukocyte interactions (Bizios R et al., 1988 **137**).

1.9.4 Regulation of the Blood Coagulation Cascade

One of the most important functions of the endothelium is to maintain the balance between thrombosis and fibrinolysis. Intact endothelium sustains blood in its fluid state by regulating platelet and coagulation activation (Figure 1.5). Platelet activity is controlled by the endothelium through the release of vasoactive mediators, prostacyclin (PGI_2) and nitric oxide (NO). The effects of PGI_2 release is mediated by G-protein coupled receptor signalling on the platelets which results in an increase in their cAMP content which inhibits their activation (Michiels C, 2003 **138**).

Figure 1.5 Regulators of the coagulation cascade



NO, in a similar fashion to PGI_2 interacts with platelets to cause reduced adhesion and aggregation at the site of injury (Tschudi MR et al., 1996 **139**).

Endothelium controls coagulation by targeting active thrombin. Antithrombotic tissue factor pathway inhibitor (TFPI) is a serine protease inhibitor which is primarily expressed by endothelial cells and to a smaller extent by platelets and leukocytes, and is considered the only known modulator of TF (Lwaleed BA et al., 2006 **140**). Its mechanism of action targets the TF-fVIIa complex and this subsequently inactivates excessive FX and FIX (Tanaka KA et al., 2009 **115**). Direct inhibitory activity results from production of the natural anticoagulant, vitamin K-dependent, protein C. This degrades the cofactors of the tenase and prothrombinase complexes (Dahlbäck B et al., 2005 **141**). Indeed, thrombin negatively regulates itself when bound to endothelial cell surface thrombomodulin (TM) since this activates protein C (APC) which results in the proteolytic cleavage of FVIIIa and FVa (van der Wouwer M et al., 2004 **142**). This is supported by the activation of protein S which is a vitamin K-dependent plasma protein (Dahlbäck B, 2005 **143**).

Regulation of thrombin also takes place by specific thrombin inhibitors, particularly antithrombin III (AT-III) a serine protease inhibitor (serpin) whose action is mediated by heparinoids (van't Veer C et al., 1997 **144**). AT-III can also degrade and expression of heparan sulphate (a glycosaminoglycan) on cell surfaces provides a substrate for antithrombin. Antithrombin adheres to these factors resulting in inactivation due the conformational change of these proteins. **(**van't Veer C et al., 1997 **145**).

Lastly, targeting the final stage of coagulation, which is the formation of the fibrin clot, is mediated by endothelial cell derived tissue-plasminogen activator (t-PA), the main enzymatic activator of the fibrinolytic system. The role of t-PA is to cleave plasminogen, an inactive protein synthesized in the liver, into its active form, plasmin. Plasmin, a serine protease, degrades fibrin and factors V and VIII (Tanaka KA et al., 2009 **115**). More specifically, fibrin expresses positively charged lysine residues which attract t-PA and plasminogen. As a result, plasmin is generated and incorporated into the fibrin, leading to clot dissolution (Esmon CT, 2003 **146**). TM, in antithesis to its anticoagulant nature, also promotes thrombin-activatable fibrinolysis inhibitor (TAFI) which removes the fibrin lysine residues. This procedure, although it reduces plasmin activation, does not terminate clot lysis (Bajzar L et al., 1996 **147**). However, at sites of severe inflammation, the fibrinolytic system is effectively controlled by serine protease inhibitor, plasminogen activator inhibitor-1 (PAI-1) by neutralizing t-PA in plasma (Juhan-Vague I et al., 2003 **148**).

1.10 Indirect Effects of DSA on the Donor Endothelium

In addition to the direct effects that DSA has on the transplant endothelium, specific subclasses of antibodies can activate complement with devastating effects to the graft (Wehner J et al., 2007 **81**). Here, complement acts as an effector of the antibody response, mediating inflammation. Complement is considered the pivotal pathway mediating HAR and AAMR due to its ability to coordinate both innate and adaptive immune responses (Kumar V et al 2006 **149**).

1.10.1 The Complement Cascade

Complement is an elaborate system of at least 35 molecules, which function as activators, receptors (Table 1.5) and regulators (Table 1.6) and was first described in the late 19th century as a heat labile bactericidal "alexin" before being redefined by Paul Ehrlich to its modern nomenclature (Ehrnthaller C et al., 2011 **150**). Its pathogenic power derives, firstly, from the generation of biologically active split products through a series of enzymatic reactions. Secondly, these split products have the ability to orchestrate and amplify responses from platelets and inflammatory cells, as well as to stimulate direct lysis and apoptosis of target cells (Wehner J et al., 2007 **81**). Currently, there are three established pathways (classical, lectin, alternative) to complement activation, (Figure 1.6) which ultimately result in three main events: C3 and C5 convertase formation and the assembly of the terminal membrane attack complex (MAC) (Zipfel PF et al., 2009 **151**).

Table 1.5 Proteins of the complement cascade

Pattern recognition	**Pathway**	**Function**
C1q	CP	Forms the C1 complex; recognizes CRP, PAMP, IgG/IgM
Collectins (MBL, Collectin 11)	LP	Recognizes CRP and some IgM and IgG
Ficolins (M, L, H)	LP	Recognize carbohydrate patterns
Properdin	AP	Recognizes PAMP; stabilizes AP convertases
Proteases	**Pathway**	**Function**
C1r	CP	Part of the C1 complex; cleaves C1s
C1s	CP	Part of the C1 complex ; cleaves C4 and C2
MASP-1	LP	Binds to collectins/ficolins; cleaves C2; may cleave C3;
MASP-2	LP	Binds to collectins/ficolins; cleaves C4 and C2
MASP-3	LP	Undefined; binds to MBL/ficolins; does not cleave C4/C2; MASP-2 inhibition
C2	CP/LP	Cleaves C3
Factor B	AP	Part of the AP convertase; cleaves C3
Factor D	AP	Cleaves factor B to form the AP convertase
Factor I	CP/LP/AP	Degrades C3b and C4b

CL=Classical Pathway

LP=Lectin Pathway

AP=Alternative Pathway

Table 1.5 continued

Complement Components	**Pathway**	**Function**
C3	CP/LP/AP	Generates C3a, C3b, iC3b, C3dg, C3d; forms AP convertase and all C5 convertase
C4	CP/AP	Generates C4b; part of the CP/LP convertase
C5	CP/LP/AP	Generates C5a and C5b enters the TCC
C6	TP	Assembly of C5b-9
C7	TP	Assembly of C5b-9
C8	TP	Assembly of C5b-9; initiation of pore formation
C9	TP	Assembly of C5b-9; undergoes polymerization for formation of lytic pore
Receptors	**Pathway**	**Function**
CR1(CD35)	CP/LP/AP	Binds C3b/iC3b/C4b/C4d; induces phagocytosis; cofactor for factor I(fI)
CR2 (CD21)	CP/LP/AP	Binds iC3b/C3d/C3dg; B cell regulation
CR3 (CD11b/CD18)	CP/LP/AP	Binds iC3b; induces phagocytosis
CR4 (CD11c/CD18)	CP/LP/AP	Binds iC3b; induces phagocytosis
C3aR	CP/LP/AP	Binds C3a; promotes inflammation
C5aR(CD88)	CP/LP/AP	Binds C5a; promotes inflammation
C5L2	CP/LP/AP	Binds C5a/C5adesArg
CRIg	CP/LP/AP	Binds iC3b/C3c; induces phagocytosis; regulates C5 convertases
cC1qR	CP/LP/AP	Binds C1q; induces phagocytosis
gC1qR	CP/LP/AP	Binds C1q; induces phagocytosis; inhibits complement activation
C1qRp	CP/LP/AP	Binds C1q; induces phagocytosis

CL=Classical Pathway TP=Terminal Pathway

LP=Lectin Pathway

AP=Alternative Pathway

1.10.2 Complement Activation by Antibodies

1.10.2.1 The Classical Pathway (CP)

The classical pathway of complement activation is dependent on circulating antibody bound to antigen. Antibodies provide the binding site for C1q (as part of the C1 complex), which, as a pattern recognition molecule, also has the ability to bind pathogens either directly or indirectly via interaction with C-reactive protein (CRP) which has itself bound pathogens (Gaboriaud C et al 2004 **152**). Only IgG and IgM have the potential to bind to the globular domains of C1q resulting in full complement activation (Colvin RB et al., 2005 **9**). Binding of C1q to the Fc tail of the antibody, leads to activation of C1r and cleavage of C1s. C1s enzymatically cleaves C4 and C2 to form C4a, C4b, C2a, and C2b fragments. The soluble anaphylatoxin C4a and the small fragment C2b remain in fluid phase and diffuse away. The larger fragment C4b forms ester or amide bonds to nearby proteins or carbohydrates and with C2a, generates the C3 convertase, C4b2a (Murata K et al., 2009 **153**). With the formation of the C3 convertase, the CP enters the common step of C3 activation which results in the cleavage of C3 to generate anaphylatoxin C3a and opsonin C3b. Addition of C3b to the C4b2a gives rise to the C5 convertase (C4bC2aC3b) and activation of the terminal pathway (Koscielska Karsprzak K et al., 2013 **154**).

1.10.3 The Lectin Pathway (LP)

Activation of the lectin pathway relies upon the recognition and binding of pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by lectin proteins. There are three main proteins associated with this pathway: mannose binding lectin (MBL) (Reid KB et al., 1994 **155**), ficolin-L and ficolin-H (Fujita T et al., 2004 **156**). However, ficolin-M which is a less well characterized protein, has been shown, like its other family members, to be capable of activating the lectin pathway (Liu Y et al., 2005 **157**). MBL and the ficolins are C-type lectins, composed of collagen and globular domains, similar to C1q. However, they form carbohydrate recognition domains (CRD) and bind to N-acetyl glucosamine and mannose structures (Carroll MC, 2004 **158**). MBL along with the proenzymes MBL-associated serine proteases, MASP-1 and MASP-2 (themselves resembling C1r and C1s) form a complex resulting in cleavage of C2 and C4. In a similar manner to the

events of the CP, the convertase C4b2a is formed leading to C3 and C5 cleavage (Ehrnthaller C et al., 2011 **150**). A third serine protease MASP-3 has been identified, but its role is less well defined. However, it has been associated with inhibiting MASP-2-mediated cleavage of C4 and C2 (Dahl MR et al., 2001 **159**).

Traditionally, the MBL pathway has not been associated with AMR, nonetheless there is evidence for MBL binding to IgM (McMullen ME et al., 2006 **160**) and some classes of IgG (Wasowska BA, 2010 **94**). The exact mechanism of how MBL binds to the carbohydrate portion of the antibody is not known (Wasowska BA, 2010 **94**). Evidence that alloantibodies activate complement through MBL has come from a murine model of AMR (Murata K et al., 2007 **161**). Results indicated that in MBL-KO mice C4d deposition (as a measure of complement activation) was decreased, even when animals were reconstituted with high doses of complement fixing IgG2a and IgG2b. These data support a role for MBL interacting with C1q resulting in the activation of complement. Data from human studies is limited, however a study in 2007 found a correlation between low serum MBL and superior graft survival (Berger SP et al., 2007 **162**). Finally, in humans co-localisation of C4d in kidney biopsies, with peritubular capillary ficolin-H and IgM (Imai N et al., 2006 **163**) as well as MASP-1 (Sund S et al., 2003 **164**) suggest a possible link between MBL pathway and AMR.

1.10.4 The Alternative Pathway (AP)

The alternative pathway, similarly to the LP and in contrast with the CP, can proceed in an antibody-independent manner. It is activated by the recognition of specific structures such as the foreign cell membranes of bacteria, yeasts and viruses as well as CRP, polysaccharides and apoptotic tissue (Thurman JM et al., 2006 **165**). AP activation is initiated in plasma with the spontaneous hydrolysis of C3, (Muller-Eberhard HJ, 1988 **166**). Through this fluid phase "tick over" mechanism, factor B becomes accessible to protease factor D. This cleavage generates Ba and Bb fragments. The latter (Bb) remains attached to C3(H_2O) forming the alternative pathway convertase C3(H_2O)Bb (Bexborn F et al., 2007 **167**). Properdin, a pattern recognition molecule itself, further stabilizes the convertase complex (Harboe M et al., 2008 **168**).

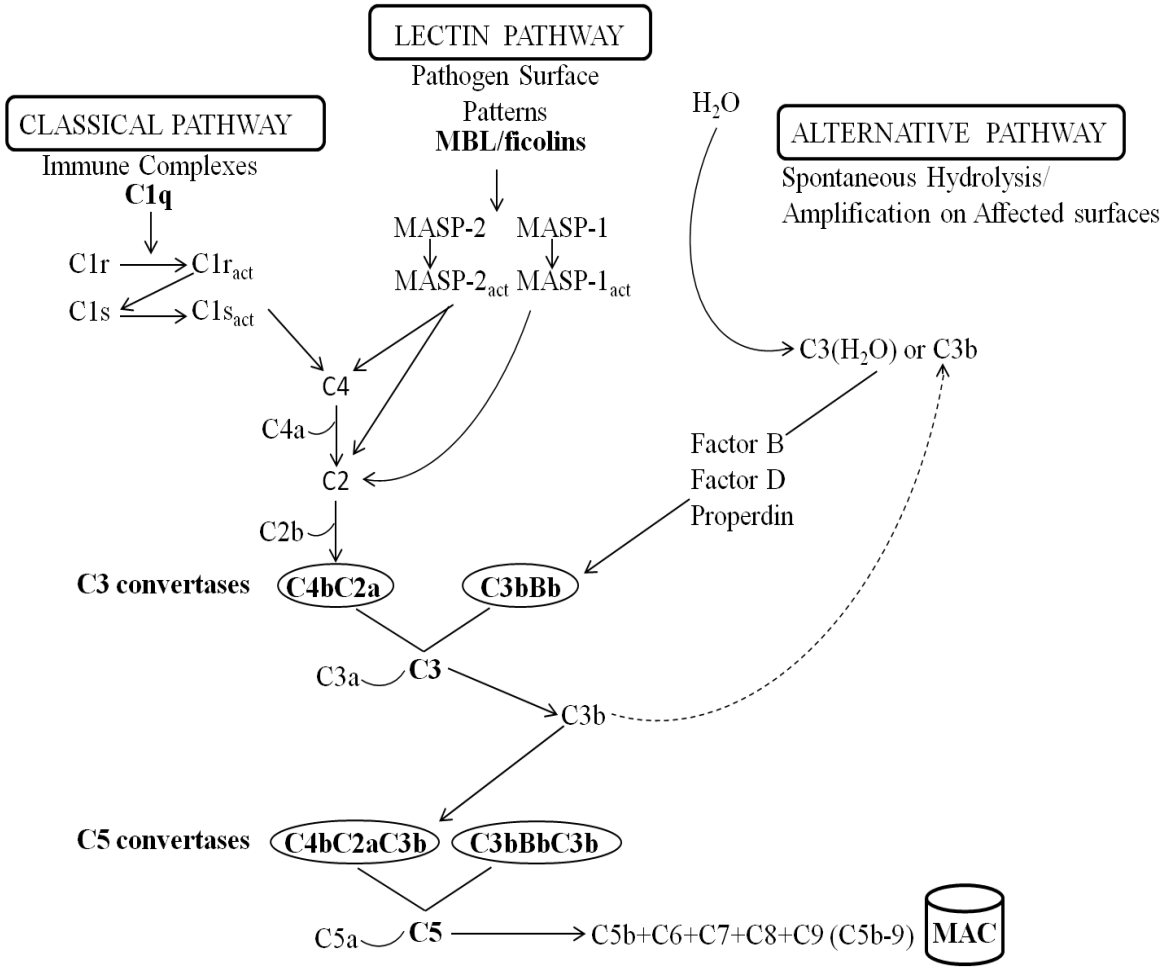
The AP is highly relevant to transplant rejection whether alloantibody is present or not since complement can be activated on surfaces or in the fluid phase, by the

amplification loop (Lutz HU et al., 2006 **169**). Initial activation of the classical and lectin pathways gives rise to C3b, which along with factors B, D and properdin generates the convertase C3bBb. This results in more C3 cleavage (more C3b) and the generation of C5 convertase. Indeed, the majority of C3b is attributed to this mechanism of generation (Harboe M et al., 2004 **170**). The huge contribution of this loop is demonstrated upon analysis of the transplant biopsies where C4b and C3b products are detected more readily than alloantibody, even when titres are extremely high (Wehner J et al., 2007 **81**). More support for the crucial role of the alternative pathway in transplant rejection, comes from the fact that, undoubtedly, the classical and lectin pathways require engagement of the AP for effective tissue injury (Thurman JM et al., 2006 **165**).

1.10.5 The Terminal Pathway

The final and common stage of all three activation pathways is the formation of membrane attack complex (MAC, C5b-9), also known as the terminal complement complex (TCC) which is responsible for complement's lytic activity (Podack ER et al., 1984 **171**). If complement activation proceeds beyond cleavage of C3, into C3a and C3b, then additional C3b binds to the C3 classical or alternative convertases. As a result, the C5 convertases formed by the CP and AP, C4bC2aC3b and C3bBbC3b respectively, are formed whose role is to cleave C5 into C5a and C5b. C5b marks the beginning of a protein assembly process leading to the initiation of the terminal pathway (Muller-Eberhard HJ, 1986 **172**). C5b along with complement components C6, C7, C8 and C9 promote lysis and cell death. C9 is the prevalent molecule, since it undergoes polymerization to generate a pore-like structure deposited on affected surfaces (Cole DS et al., 2003 **173**). This tubular structure disrupts phospholipid bilayer surfaces either by creating a "leaky patch" (Esser AF, 1991 **174**), without forming a pore, or, the formation of a rigid hydrophilic channel (pore) penetrating the membrane and thereby disrupting the cell (Bhakdi S et al., 1991 **175**). In AMR, large amounts C5b-9 trigger disruption of vascular surfaces but sublytic amounts of C5b-9 can be enough to activate the endothelium. The latter can have more devastating effects than cell lysis itself (Wehner J et al., 2007 **81**).

Figure 1.6 The complement cascade



1.10.6 Effects of the Complement System

The main effector function of complement, being an important part of innate immunity, is defense against infections through proinflammatory responses, chemotaxis and opsonization (Ricklin D et al., 2010 **176**). In addition, complement not only initiates inflammation, but also clears it, by removal of immune complexes and apoptotic cells (Flierman R et al., 2007 **177**). Furthermore, acting as a bridge between innate and adaptive immune responses, it triggers and orchestrates B cell (Marsh JE et al., 2001 **178**; Carroll MC, 2008 **179**) and T cell responses (Heeger PS et al., 2011 **180**). Extensive work has been carried out in our department signifying the fundamental role of complement in the T cell alloresponse (Pratt J et al., 2003 **181**; Peng Q et al., 2006

182; Zhou W et al., 2006 **183**; Sacks SH et al., 2012 **184)**. Furthermore, it is now known that complement is a multifunctional system whose role extends far beyond pathogen clearance. Complement plays an important role in homeostasis, interacts with other biological systems, such as Toll-like receptors (TLRs) (Hajishengallis G et al, 2010 **185**) and coagulation (Markiewski MM et al., 2007 **186**) and participates in tissue repair (DeAngelis RA et al., 2006 1**87**) and metabolism (Raisz LG, 2001 **188**). The main effector mechanisms of complement are summarized in figure 1.7.

1.10.7 Regulation of the Complement Cascade

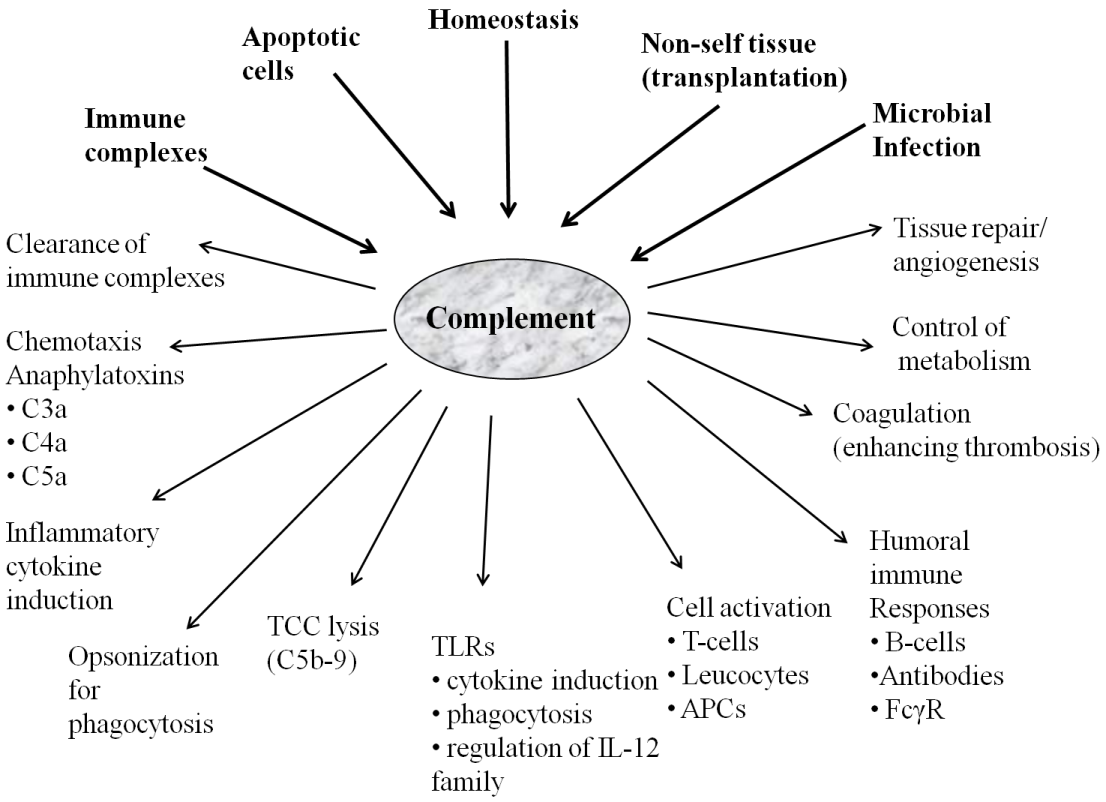
Complement is a potent network with multiple effector mechanisms that can be directed against the host. For that reason, it is crucial that its activity is tightly regulated as uncontrolled activation of the complement cascade would result in consumption of its factors, and lead to damage of healthy tissue. Evidence for this requirement is the existence of a large number of regulators, categorized into two classes; fluid phase and membrane bound regulators (Table 1.6) (Zipfel PF et al., 2009 **151)**.

1.10.7.1 Soluble Regulators

A number of fluid phase regulators are found in human plasma. C1 inhibitor (C1-INH) inhibits both the classical pathway serine proteases, C1r and C1s as well as lectin pathway MASP-1 and MASP-2 (Ehrnthaller C et al., 2011 **150**). C4BP, with factor I (fI), inhibits both the classical pathway (CP) and alternative pathway (AP) convertases (Ricklin D et al., 2010 **176**). Factor H (fH) and the factor H-like protein 1 (FHL-1/reconectin) act with fI to target the AP convertase by degrading C3b (a process known as cofactor mediated cleavage) or alone to remove the Bb fragment from the C3 convertase (a process known as decay acceleration) (Józsi M et al., 2008 **189**). Upon cleavage, C3 and C5 anaphylatoxins are formed, which carboxypeptidase N rapidly inactivates to less active C3a desArg and C5a desArg respectively (Noris M et al., 2013 **190**). Furthermore, factor H-related protein 1 (FHR-1) has a distinct function to fH. It targets the C5 convertase, and therefore inhibits the formation of MAC (Heinen S et al., 2009 **191**). Similarly, clusterin and vitronectin (S-protein) target the terminal pathway by binding to C7 and C5b-9 respectively, thereby preventing MAC formation and integration into membranes (Noris M et al., 2013 **190**). It should be noted that, many of these fluid phase control proteins such as fH, FHL-1, FHR-1, C4BP, vitronectin and

clusterin are also found on cell surfaces to aid and maintain complement regulation (Zipfel PF et al., 2009 **151).**

Figure 1.7 Effector mechanisms of the complement system



1.10.7.2 Membrane Bound Regulators

Regulatory proteins attached to cells include membrane cofactor protein (MCP/CD46) which acts as a cofactor for fI and induces C3b and C4b cleavage (Thurman JM et al., 2011 **192**). Decay accelerating factor (DAF/CD55) is another surface bound protein and it regulates complement activity by binding to C3b dissociating C2a from the CP and LP convertase and Bb from the AP convertase (Hourcade DE et al., 2002 **193**). CR1 (CD35) is widely distributed on the surface of erythrocytes (RBC), leukocytes and kidney glomerular basement membrane and is considered to be the most powerful surface bound complement regulator. CR1 displays both decay acceleration as well as

cofactor activity for factor I. It promotes cleavage of C3b to iC3b, C3c and C3dg as well as C4b to iC4b, C4c and C4d (Kim DD et al., 2006 1**94**). This is of particular importance since these fragments are ligands for cells with complement receptors CR1, CR2, CR3 and CR4. For this reason, endothelial injury mediated by complement is accompanied by extensive leukocyte infiltration which mediates phagocytosis and clearance (Murata K et al., 2009 **153**). CD59 (protectin) inhibits cell lysis by preventing polymerization of C9 for MAC formation and consequently the integration of pores into the cell membrane (Morgan BP, 1995 **195**).

1.10.7.3 Rodent Complement Regulators

The importance of complement regulator proteins in protecting the host from homologous complement attack is supported by a wealth of experimental data. This has resulted extensive knowledge of complement regulators in mice and rats. In mice, CR1 is expressed by B cells, phagocytes and resting granulocytes and in contrast to humans it is not expressed by rodent T cells (Kinoshita T et al., 1988 **196**). Functionally, mouse CR1 has similar regulatory function to human CR1 acting as a C3 receptor thereby protecting host tissue from complement-mediated damage (Molina H et al., 1992 **197**). Furthermore, human CR1 is functional in rats as indicated by experiments using human soluble CR1 in a rat model to reduce immune complex-mediated vasculitis (Mulligan MS et al., 1992 **198**). Interestingly, Molina H et al. identified a C3b-derived protein, p65, which is widely distributed in a variety of tissue such kidney, brain, lung, pancreas and skin (Molina H et al., 1992 **197**) as well as lymph nodes and expressed on peritoneal macrophages and erythrocytes (Li B et al., 1993 **199**). This additional molecule has identical biochemical properties to human regulatory DAF and MCP (Kim YU et al., 1995 **200**). It acts as a cofactor for factor I-mediated cleavage of C3b and C4b and targets the CP and AP convertases acting through decay-acceleration. This molecule was named Crry (Complement receptor 1-related protein Y) and it was also found to be expressed on platelets, neutrophils and endothelial cells (Quigg RJ et al., 1995 **201**). Crry in mice has been found to be involved in $CD4^+$ T cell activation, acting as a co-stimulatory molecule (Centero-Fernandez E et al., 2000 **202**).

Rats and mice also express an analogue of human DAF (Hinchcliffe et al., 1998 **203**). In the rat, DAF is widely distributed on the endothelium, erythrocytes, platelets and B cells

as well as numerous organs such as the kidney, lungs, liver and spleen (Spiller OB et al., 1999 **204**) and functions to inhibit complement across species. For example, rat DAF has been found to inhibit the CP-mediated haemolysis of antibody sensitised cells incubated with mouse or human sera (Harris CL et al., 2000 **205**).

The rodent homologue of MCP has also been identified. Indeed, the structures for mouse and rat MCP are very similar (Mead R et al., 1999 **206**). Mouse MCP is expressed in testis whereas in rats it is found in testis and the acrosome of developing spermatozoa (Mizuno M et al., 2004 **207**). This contrasts with the more widespread tissue distribution of MCP in humans (Liszewski MK et al., 1992 **208)**. Again, species cross-reactivity has been shown. Mouse MCP inhibits cell lysis by human complement (White DJ et al., 1992 **209**).

Membrane bound CD59 has also been described in the rat. Tissue expression is similar to that in humans with wide distribution on all circulatory cells and endothelium of arteries, veins and capillaries. CD59 is also expressed in organs such as the liver, kidney, heart and lungs (Funabashi K et al., 1994 **210**). Expression of rat CD59 on Chinese hamster ovary (CHO) cells was able to successfully inhibit cell lysis in pig, rabbit or human sera. The degree of protection against human serum was highest showing minimum evidence for species selectivity (Rushmere NK et al., 1997 **211**). More recently, murine CD59 protein has been found to be encoded by two homologous genes, giving rise to CD59a and CD59b protein products. CD59a has a broad tissue distribution in contrast to CD59b which is found in the testis (Harris CL et al., 2003 **212**).

Table 1.6 Regulators of the complement cascade

Soluble Regulators	**Pathway**	**Function**
C1-INH (SERPIN 1)	CP/LP	Inhibits serine proteases C1r, C1s and MASPs
Factor H	AP	Cofactor for factor I; decay acceleration activity for C3 convertases
FHL-1 (reconectin)	AP	Cofactor for factor I; decay acceleration activity for C3 convertases
FHR-1	AP	Inhbition of C5 cleavage
Carboxypeptidase-N	CP/LP	C3a and C5a degradation
C4BP	CP/LP	Cofactor for factor I; decay acceleration activity for CP/LP convertases
Clusterin	TP	Prevents MAC assembly
Vitronectin (S-protein)	TP	Prevents MAC assembly
Membrane Bound Regulators	**Pathway**	**Function**
CR1 (CD35)	CP/LP/AP	Cofactor for factor I; decay acceleration activity for all C3 convertases
CRIg	CP/LP/AP	Binds iC3b/C3c; induces phagocytosis; regulates C5 convertases
MCP (CD46)	CP/LP/AP	Cofactor for factor I degradation of all C3 convertases
DAF (CD55)	CP/LP/AP	Decay acceleration activity for all C3 convertases
CD59	TP	Prevents MAC assembly

CL=Classical Pathway

LP=Lectin Pathway

AP=Alternative Pathway

TP= Terminal Pathway

1.10.8 Complement Activation of Leukocytes and Endothelial Cells

An important mechanism by which acute graft injury is mediated is the accumulation of inflammatory cells at the site of injury. The presence of neutrophils, monocytes and macrophages is typical of AMR (Murata K et al., 2009 **153**) and the release of large quantities of cytokines such as IL-1, IL-12, TNF-α and IFN-γ by activated leukocytes has been demonstrated in graft impairment (Jose MD et al., 2003 **213**; Sund S et al., 2004 **214**). These events are largely the result of leukocytes and endothelial cells responding to the products of the complement activation cascade.

Proinflammatory anaphylatoxins, C3a and C5a, are highly potent chemoattractants and their effects are mediated by the expression of surface receptors on leukocytes, C3aR and C5aR (CD88) (Haas PJ et al., 2007 **215**). A second receptor, C5L2, has also been found to bind C5a with high affinity, although its function remains controversial (Li R et al., 2013 **216**). C3a is spasmogenic and upon interaction with macrophages, prostaglandin E_2 (PGE_2) is released, inducing chemotaxis and cytokine production (Fischer WH et al., 1999 **217**). C5a, on the other hand, is considered to be the most potent anaphylatoxin of the two with additional functions to chemotaxis, such as influencing proinflammatory responses in leukocytes, promoting oxidative burst and phagocytosis (Mollnes TE et al., 2002 **218**).

Initial activation of leukocytes by C3a and C5a induces upregulation of complement receptors CR1 and CR3. These receptors are specific for complement split products C4b, C3b, iC3b and C3d deposited on allografts further enhancing their opsonization (Wehner J et al., 2007 **81**). CR3, upon cytokine stimulation, has been shown to be involved in leukocyte trafficking and synapse formation (Ricklin D et al., 2010 **176**).

Endothelial cells are activated directly by complement due to their expression of C3aR and C5aR, resulting in increased secretion of proinflammatory cytokines and chemokines (Saadi S et al., 2001 **219**). *In vitro*, C1q engagement on human endothelial cells stimulates the release of the chemokines MCP-1 and IL-8 (van den berg RH et al., 1998 **220**). C3a and C5a effects on the capillary endothelium involve the production of cytokines (IL-1, IL-6), chemokines (CCL5, CXCL8) (Monsijon T et al., 2003 **221**) and also upregulation of adhesion molecules (E-selectin, ICAM-1, VCAM-1) (Jagels MA et al., 2000 **222**).

C5b-9 deposition has an additive effect to chemotaxis and adhesion, especially through its stimulation of IL-1, even at sublytic levels (Saadi S et al., 2000 **223**). Endothelial cells activated by the MAC, elicit proliferation signals through secretion of PDGF and bFGF (Benzaquen et al., 1994 **224**).

1.10.9 Complement and B cells

Another mechanism, by which complement promotes an antigen-specific immune response, is the stimulation of B cells through their CR1 (CD35) and CR2 (CD21) receptors. Both CR1 and CR2 form the B cell co-receptor complex in association with CD19 and CD81 (Carroll MC, 2008 **179**). C3d opsonized antigen, bound to CR1 and CR2 on the B cell co-receptor, results in an enhanced signal. Co-ligation of CR/CD19/CD81 co-receptor with the B cell receptor lowers the threshold for B cell activation by several degrees of magnitude (Carroll MC, 2004 **158**). In this manner, C3d act as an adjuvant to the B cell response (Fearon DT et al., 1996 **225**). Thus, C3d deposition on cells, such as those expressing allogeneic HLA, acts as a positive feedback mechanism enhancing a pre-existing antibody response. The link between the complement system and B cell responses was further supported by experiments carried out using C3-deficient mice. These showed impaired allogeneic IgG responses when they received fully MHC mismatched skin grafts (Marsh JE et al., 2001 **178**). In addition, complement induces an antibody response through C5aR signalling on macrophages by upregulating the expression of activating FcγRI and FcγRIII, and downregulating inhibitory FcγRIIB (Shushakova N et al., 2002 **226**). Finally, follicular dendritic cells (FDC) are crucial for B cell immunity, as they also bear CR1 and CR2 receptors. In this way, FDC can efficiently trap and retain C3d-coated antigen within the lymphoid follicles to support long term memory and antibody production (Fang Y et al., 1998 **228**).

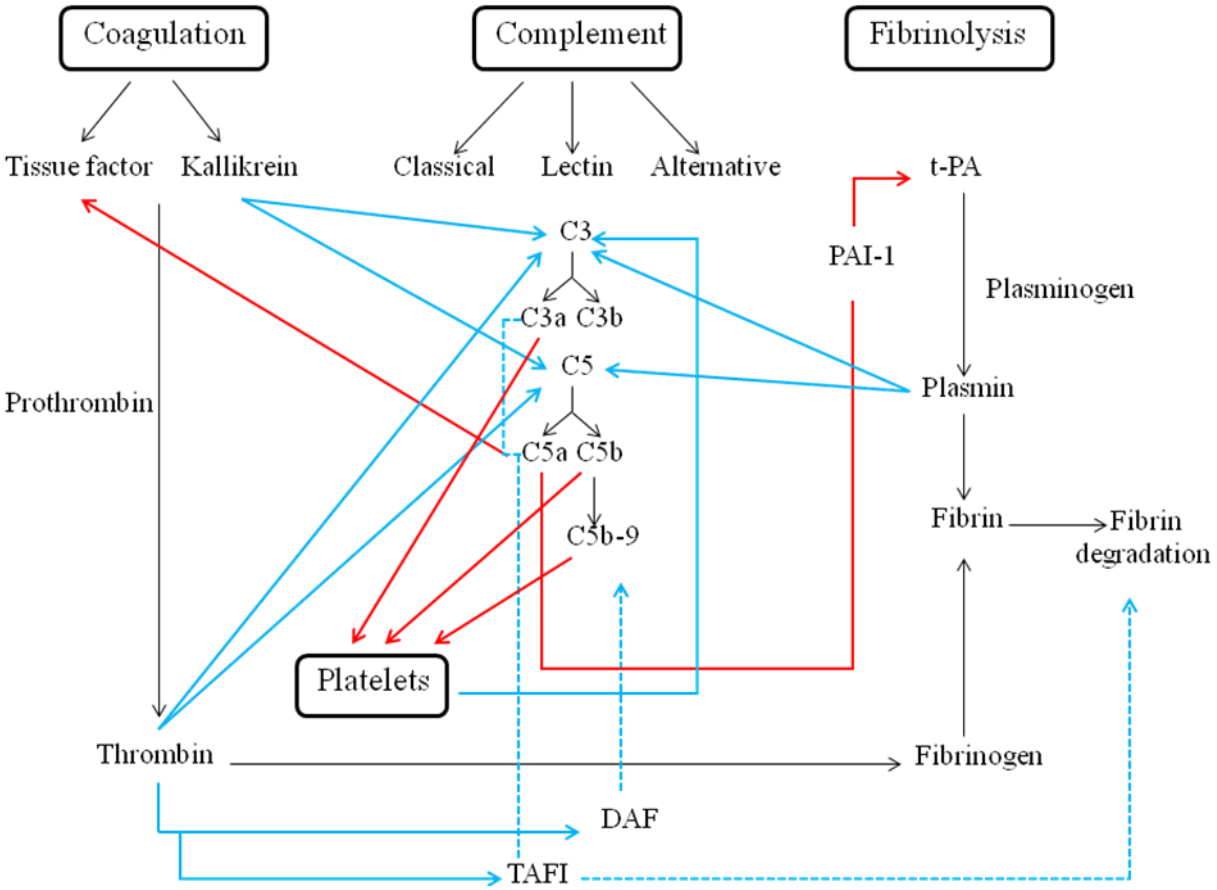
1.11 Interplay between the Complement and Coagulation Cascades during Graft Rejection

Complement and coagulation, although throught to be derived from a common ancestral pathway, have traditionally been considered to be two distinct cascades. However, there is increasing evidence for the extensive cross-talk between these two pathways, occurring at an early stage of inflammation (Amara U et al., 2010 **228**). Although this remains controversial, it is quite possible that, in most pathophysiological situations,

their activation takes place simultaneously resulting in the parallel expression of effector products. In this manner, these two pathways are able to support, amplify and even regulate each other, acting together in response to inflammation, aiming at restoring balance to a disturbed system (Markiewski MM et al., 2007 **186**). In contrast, it remains possible that one pathway precedes the other (traditionally, traditionally coagulation following complement activation, however this is now being questioned, as mentioned below). This topic remains controversial, with evidence supporting both hypotheses.

Communication between the complement and coagulation cascades appears to be extensive (Figure 1.8), which may be of no surprise considering the similarities they share. First of all, both systems act in response to an inflammatory stimulus and both act locally where altered or (in the case of rejection) foreign surfaces cause their activation. Furthermore, it is of pivotal importance, that both cascades have an initiation and amplification stage. Indeed, interactions with target cells, or directly with each other, often results in amplification of one system by the other. Finally, the potency of these serine protease cascades is evident by the fact that both of them require tight regulation by a variety of control molecules. If present at the same time, this can result in simultaneous regulation of both systems (Oikonomopoulou K et al., 2012 **229**).

Figure 1.8 Complement-coagulation cross-talk (adapted from Oikonomopoulou K et al., 2012 ***197****)*



** blue-coloured lines refer to amplification of complement by coagulation, red-coloured lines refer to amplification of coagulation by complement and dotted lines refer to inhibitory actions of the pathway.*

1.11.1 Coagulation initiating the Complement Cascade

Although both systems respond rapidly at the site of inflammation, the concept of coagulation potentially initiating complement has recently been addressed and supported by an extensive body of work. This evidence challenges the dogma that it is complement activation that invariably is responsible for the activation of coagulation cascade. It is well recognised that clotting factor XIIa is capable of initiating the C1 complex formation, thereby activating the complement classical pathway (Ghebrehiwet

B et al., 1981 **230**), the primary pathway involved in AMR. In addition, it is interesting that, C1-esterase inhibitor not only inhibits all three complement pathways, but also inhibits the intrinsic coagulation cascade (Davies AE 3rd, 2004 **231**). These two observations reveal the close interaction of the two cascades, as well as the necessity to control complement early in its activation cascade, well before the convertase formation.

Particular interest has derived from the ability of certain coagulation enzymes to directly cleave C3 and C5 revealing a bypass mechanism for complement activation. *In vitro* studies showed that thrombin, plasmin, factors IXa, Xa and XIa were all able to cleave C3 and C5, giving rise to anaphylatoxins with competent chemoatraction abilities (Amara U et al., 2010 **228**). These results have been additionally supported by liver studies, in which thrombin and plasmin were capable of activating C3 in experimental systems where all three complement activation pathways had been disrupted (Clark A et al., 2008 **232**). Finally, in a lung injury model, using $C3^{-/-}$ mice, thrombin was able to directly cleave C5 and generate biologically active C5a anaphylatoxin (Huber-Lang M et al., 2006 **233**). Krisinger et al. extended these studies by identifying a new site where thrombin cleaves C5, and confirming the strong lytic activity of C5b-9 generated by thrombin cleavage (Krisinger MJ et al., 2012 **234**).

Additionally, thrombin may indirectly interfere with complement activation through its ability to activate platelets. Thrombin, is considered one of the most potent stimulators of platelets and therefore essential for secondary haemostasis. Platelets, although somewhat ignored in the field of graft rejection, have been of increasing interest since they influence complement activation by expressing receptors for C1q. Upon binding C1q, the initiator of the classical pathway of complement activation, P-selectin expression is induced which binds C3b. Through this interaction, platelets prolong the lifespan of the C3b (Ekdahl KN et al., 1999 **235**) and activate the alternative pathway (Nilsson-Ekdahl K et al., 2001 **236**). Thus, the activation of platelets by thrombin leads to complement activation and complement-mediated injury.

Finally, at sites where both cascades are activated, it has been shown that thrombin activation of PAR-1 negatively regulates complement activation by inducing DAF expression, in diseases such atherosclerosis and vasculitis (Lidington EA et al., 2000

237). In addition, although TAFI is associated with inhibition of fibrinolysis, it can also inactivate C3a and C5a (Oikonomopoulou K et al., 2012 **229**).

1.11.2 Complement initiating the Coagulation Cascade

Although the above evidence strongly suggests an initiating role for the coagulation cascade, extensive data support the procoagulant properties of complement, which may therefore precede the coagulation pathway. Recently, the MBL pathway has been implicated in a novel role in activation of the clotting cascade. In an animal model of arterial thrombosis, MASP-1/-3 knockout (KO) mice had significantly reduced thrombogenicity, with MASP-1 in particular exhibiting thrombin-like activity (La Bonte LR et al., 2012 **238**). Krarup et al showed similar findings, using $C3^{-/-}$ mice, in which he was able to demonstrate the ability of MASP-2 to activate prothrombin in a similar manner to fXa (Krarup A et al., 2007 **239**).

However, possibly the most significant studies showing profound activation of coagulation by complement involve C3a and C5a. C3a and C3a desArg can directly induce platelet adhesion and aggregation (Volanakis JE et al., 1989 **240**). C5a, on the other hand, has multiple effects on both endothelial and inflammatory cells. C5a has the ability to modulate endothelial cell surfaces to favour clot formation. C5a in combination with alloantibodies on the endothelial surface promotes the shedding of heparan sulfate, which is responsible for the preservation of an anti-coagulant environment (Platt JL et al., 1991 **241**). This finding has been strongly associated with HAR and AMR (Colvin RB et al., 2005 **9**). C5a enhances the propensity for intravascular thrombosis not only by inducing TF on endothelial cells (Ikeda K et al., 1997 **242**), but also on leukocytes (Muhlfelder TW et al., 1979 **243**). Furthermore, C5a causes upregulation of PAI-1 on leukocytes, which abolishes the enzymatic activity of t-PA (Wojta J et al., 2002 **244**). It also induces IL-8 on endothelial and blood cells, and IL-6 on platelets which strongly promotes fibrin formation (Guo RF et al., 2004 **245**). C5a thereby shifts the balance towards a procoagulant clot forming phenotype.

The formation of MAC (C5b-9) reinforces activation of the coagulation cascade since stimulation of platelets with C5b-9 results in further activation of platelets by release of their granules (Ando B et al., 1988 **246**) and TF bearing MPs (Sims PJ et al., 1988 **247**). Since platelets are the primary surface at which the prothrombinase complex occurs, MAC interactions propagate thrombin generation. Finally, MAC deposition on

endothelial cells disrupts the monolayer exposing the subendothelium to the blood, initiating coagulation (Bossi F et al., 2004 **248**).

Complement can positively regulate coagulation where C4BP has been deposited onto affected surfaces. Here C4BP forms a complex with Protein S, a cofactor for the anticoagulant APC system. This binding results in the loss of its cofactor ability, therefore decreasing Protein C inhibitory activity. As a result, complement can be responsible for increasing the tendency for thrombus formation (Dahlbäck B et al., 2005 **141**).

In conclusion, it is clear that antibodies against antigens on transplanted organs are a serious obstacle to short and long term graft survival. AMR is a dynamic process which is characterized by complement activation as well as complement-independent mechanisms. As a result, antibody-mediated mechanisms such as endothelial activation, inflammatory alterations and intravascular coagulation make AMR a complex challenge to overcome, and therefore the optimal strategy for effective prevention has been far from clear. The effector mechanisms involved in humoral rejection are summarized in figure 1.9.

Antigen Cross-Linking
Proliferation
Exocytosis of vWf
Expression of adhesion molecules
Platelet aggregation
Tissue factor-Activity
Inflammation-
Intravascular Thrombosis

Complement
Proliferation
Expression of C5a/C3a
Chemoattraction
C5b-9 (MAC)
Lysis of target cell

Fc Receptor Activation
Interaction with:
PMN,
NK Cells,
Macrophages
Apoptosis of the antibody-coated cell

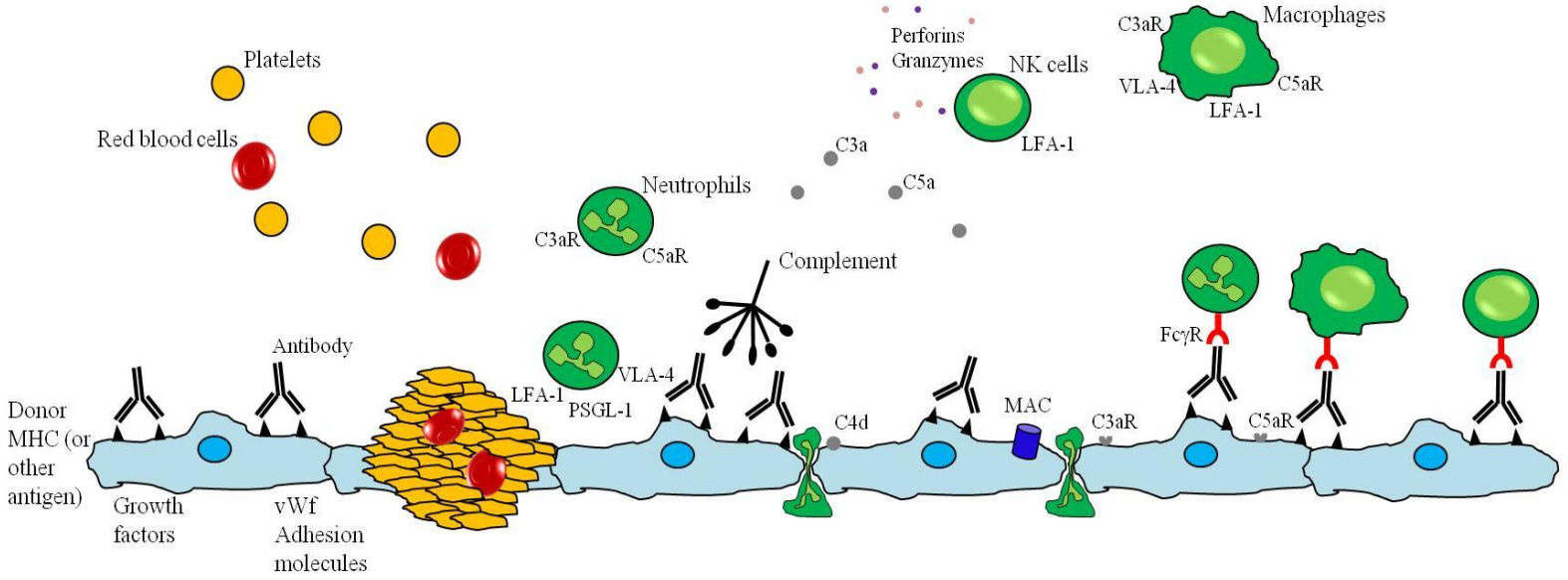


Figure 1.9 Diagram illustrating the major antibody-mediated processes leading to graft rejection

1.12 The Role of T cells in Clinical Transplantation

Cellular rejection involving T cells is the most common form of allograft rejection. Cellular rejection is initiated through the process of allorecognition, where T cells recognize donor antigen (major and/or minor histocompatibility) (Ingulli E, 2010 **249**). Allorecognition and priming of recipient T cells is mediated through three distinct pathways. The direct pathway involves the donor's antigen presenting cells (APCs) carrying intact alloantigen from the graft to the recipient's secondary lymhoid organs, where alloreactive T cells respond to it (Nankivell BJ et al., 2010 **30**). These donor-derived APCs exist in limited numbers and eventually, although the direct pathway is responsible for the initiation of rejection, the indirect pathway of allostimulation is thought to take over in maintaining the donor-specific T cell response. Through the indirect pathway, the recipient's APCs present peptides from degraded donor MHC contributing to the activation of T cells and destruction of the graft. Eventually the indirect pathway will be the dominant pathway of allostimulation and it occurs for as long as the organ remains *in situ* (Wood KJ et al., 2012 **250**). This pathway is associated with chronically rejected grafts (Ingulli E, 2010 **249**). Finally, a more recently defined pathway of allorecognition, is the semi-direct pathway in which the host's APCs acquire and present intact donor MHC-peptide complexes as well as presenting domor peptides on their on MHC (Afzali B et al., 2008 **251**).

As a consequence of allorecognition, activation of alloreactive T cells occurs. This process is mediated by two signals: signal 1 is antigen specific and results from the engagement of the T cell receptor (TCR) with a MHC-peptide complex. Signal 1 on its own is not enough for full activation, for which a second signal is needed. Signal 2 results from a series of costimulatory molecules binding their ligands. CD28 is the best defined costimulatory molecule on T cells and binds members of the B7 family (CD80 and CD86) expressed on APCs. Signalling through CD28 is of pivotal importance since it lowers the threshold for T cell activation, is responsible for the release of cytokines for proliferation and resistance to apoptosis (Wood KJ et al., 2012 **250**). T cells also express CD154 (CD40L) which is the ligand for CD40, expressed not only on APCs (Ridge JP et al., 1998 **252**) but also B cells. The interaction of CD154 and CD40 on B cells is necessary for T-dependent B cell activation, B cell differentiation and antibody production (Tarlinton DM et al., 2008 **253**). CTLA-4 (CD152) is transiently expressed

on T cells, however, its ligation to the B7 molecules delivers an inhibitory signal (Walunas TL et al., 1996 **254**). Its tightly controlled surface expression is critical to regulating T cell immunity.

Following activation, T cells proliferate and differentiate into subsets with distinct cytokine signatures. $CD4^+$ T helper cells may largely be defined as being Th1 or Th2, however many other helper subsets exist such as Th17, Th9 and Tfh (follicular helper cells). The characteristic Th1 cytokine is IFN-γ whereas the Th2 subset secrete IL-4, IL-5 and IL-13 (Wood KJ et al., 2012 **218**). Activated cells also include cytotoxic $CD8^+$ T cells, and $CD4^+$ T cells with a suppressing role, regulatory T cells (Tregs). Tregs (naturally occurring or induced) may generate and maintain a state of tolerance *in vivo* (Wood KJ et al., 2003 **255**).

Following this elaborate process by which naïve T cells pass from the circulation to the secondary lymphoid organs, become activated and differentiate into effector T cells, they migrate to the transplanted tissue. This movement to the graft requires a variety of chemokines and integrins (such as LFA-1) binding to adhesion molecules on the graft endothelium (such as CD54/ICAM-1). These interactions facilitate the rolling and the tethering of alloreactive T lymphocytes (Briscoe DM et al., 1998 **256**). T cells extravasate through the endothelium, infiltrate the interstitium and in the case of a kidney transplant, invade the renal tubules (Robertson H et al., 2003 **257**).

T cells cause the destruction of the transplanted organ via two mechanisms: delayed type hypersensitivity (DTH) and $CD8^+$ mediated cytotoxicity. DTH involves $CD4^+$ helper T cells (Th) releasing highly proinflammatory cytokines such as IFN-γ. This results in the influx of monocytes, macrophages, neutrophils and eosinophils. Th lymphocytes are also responsible for the production of activating mediators such as nitric oxide (NO), reactive oxygen species as well prostaglandin E_2 and thromboxane (Wood KJ et al., 2012 **250**). Furthermore, activated alloreactive $CD8^+$ T cells release cytotoxic molecules, granzyme B and perforins, upon identification of their target antigens. Killing is mediated by inducing apoptosis (Barry M et al., 2002 **258**).

1.13 Immunosuppression

Due to the recipient's alloresponse to the graft, organ transplantation between non-identical individuals generally cannot be successful in the absence of aggressive and

continuous immunosuppression (Table 1.7). The immunosuppressive regimen used may differ according to the transplant centre and over time to provide an ideal balance between preventing the alloresponse whilst maintaining the ability to fight infection and malignancy. Furthermore, different immunosuppression may be used as induction and maintenance at the time of transplantation and thereafter. Absence of immunosuppression in these circumstances will lead to the destruction of the graft. Allograft failure can occur due to immunological and/or non immunological reasons. Immune causes involve acute and chronic rejection, mediated by HLA differences between the donor and recipient (Vathsala A, 2005 **259**). The identification and minimization of HLA disparities as well as the use of potent immunosuppression has enabled excellent outcomes following acute rejection. In contrast, although early renal failure has become rare, late graft loss still remains problematic (Hardinger KL et al., 2013 **260)**. In addition, non immunological complications, such as ischaemia, malignancies, hypertension, infections and calcineurin inhibitor toxicity compromise the success of long term accepted grafts (Vathsala A, 2005 **259)**.

An area which still provides an obstacle to successful transplantation, especially in chronically failed organs, is humoral rejection. AMR is difficult to treat because the mechanism is only partially understood, as reflected in the poor response to conventional immunosuppressive drugs (Hardinger KL et al., 2013 **260)** Typically, AMR is treated with plasmapheresis, intravenous immunoglobulin (IVIG) and Rituximab. However, further options are needed further investigation as long term effects regarding toxicity and efficacy are still unclear. Several clinical trials have focused their attention on complement with particular interest in Eculizumab. Eculizumab is a humanized monoclonal antibody which binds and blocks the activity of the complement protein C5. Eculizimab has been successfully used in the treatment of atypical haemolytic uremic syndrome (aHUS) (Châtelet V et al., 2010 **261)** and thrombotic microangiopathy (Chadran S et al., 2011 **262)**. In solid organ transplantation, the successful use of Eculizumab has been reported in a small number of cases of AMR in patients with high DSA (Lonze BE et al., 2010 **263**; Stegall MD et al., 2012 **264)**. Despite the promising effects shown by eculizumab, it also has drawbacks as it is administered systemically, causes a number of side effects such as nausea, fatigue, headaches, infection (Hardinger KL et al., 2013 **260)** and it has been involved in fatal immune haemolytic aneamia (Rovira J et al., 2013 **265)**.

A particular issue in the clinic has been the rise in the number of sensitised renal patients requiring transplantation. These transplants have been made possible by the introduction of increasingly aggressive immunosuppressive regimens: although sensitised recipients no longer lose their graft to HAR, their high DSA levels contribute to acute rejection episodes and associated long-term damage to the kidney. For these graft recipients intense desensitisation protocols are undertaken to avoid HAR, including plasmapheresis, IVIG and suppression of B cells and plasma cells. This process is not only time-consuming and costly, but the long term results are not yet certain. In addition, due to their high risk of AAMR, pre-sensitised patients tend to remain on the transplant list for a long time and may die while waiting for a suitable graft. These people constitute as one-third of the kidney transplant waiting list. Even if a suitable donor is found the incidence of acute rejection remains high, maybe up to 60%. In the UK, the average waiting time before receiving a donor kidney is 1156 days (3 years and 61 days) (www.organdonation.nhs.uk).

Table 1.7 Main immunosuppressive agents

Drugs	Mechanism of Action
Prednisolone (induction/maintenance)	Corticosteroid; down-regulates T cell and APC cytokine and cytokine receptor expression
Cyclosporine (CsA) (maintenance)	A calcineurin inhibitor; prevents transcription of IL-2
Tacrolimus (FK-506) (maintenance)	Inhibits calcineurin and IL-2 expression
Mycophenolate Mofetil (MMF) (Cellcept) (maintenance)	Anti-proliferative; Inhibits inosine monophosphate dehydrogenase
Azathioprine (AZA) (maintenance)	Anti-proliferative; Prodrug for mercaptopurine; inhibits purine biosynthesis and CD28 signalling
Sirolimus (Rapamycin) (maintenance)	Anti-proliferative; A inhibitor of the mammalian target mTOR; inhibits IL-2 responses
Everolimus (maintenance)	A rapamycin derivative
Basiliximab (Simulect) (induction)	Chimeric mouse-human monoclonal antibody against IL-2 receptor (CD25); blocks B7-CD28 signalling
Anti-thymocyte globulins (ATG) (induction)	Polyclonal rabbit antibodies against T cells
Alemtuzumab (Campath 1H) (induction)	Humanised monoclonal antibody against CD52; depletes T- and B-lymphocytes
Intavenous immunuglobulins (IVIG) (induction)	Polyclonal antibodies against T cells.

For this reason, more research is needed to develop anti-rejection therapy that better balances the risks and benefits. An ideal immunosuppressive strategy would successfully improve long term acceptance of the graft but limit the systemic toxicity and risk of infection in the transplant recipient which remain a major cause of morbidity and mortality, especially in this disadvantaged and common group.

1.14 Current Status of AMR and Animal Models

Interest in the role of antibodies in graft rejection dwindled after decades of research which demonstrated that alloreactive T cells were an essential and common cause of rejection episodes together with the fact that HAR could largely be avoided by not transplanting those patients with DSA and high risk of HAR. However, interest in the role of antibodies was re-ignited following the observation that a small number or renal biopsies exhibited features of "pure" AMR (Halloran PF et al., 1990 **266**).

Subsequently, increased acknowledgment of alloantibodies contributing to graft injury resulted in the terms “acute” and “chronic antibody-mediated rejection” incorporated in the Banff classification in the beginning of 2000 (Durlik M, 2013 **267**). Indeed, AMR appears to be one of the main factors in acute and chronic rejection with both *de novo* and preformed antibodies actively participating in the process.

Animal models have contributed to our understanding of AMR in a number of ways. Using immunoglobulin (Ig) KO mice, in a cardiac transplant model, Murata K et al. were able to demonstrate that complement was secondary to alloantibody production, such that deposited C3d and C4d were absent from grafts in KO mice compared to their transplanted wild type counterparts (Murata K et al., 2007 **161**). With the same model, passive transfer of antibody against donor MHC restored complement activation and led to graft rejection (Wasowska BA et al., 2001 **268**).

Studies in mice with specific deficiencies in complement have further illuminated the role of complement in AAMR (Winn HJ et al., 1973 **269**) although it should be noted here that a number of wild type mouse strains have complement deficiencies (e.g. C5 deficiency in A/J, AKR, B10D2, and DBA/2) which should be taken into account when interpreting the results (Chong AS et al., 2013 **270**). In addition, research into HAR using xenotransplantation models has been informative, in particular regarding the role of complement regulatory proteins and their ability to protect endothelial cells from antibody-mediated damage (Morgan BP, 1995 **271**). McCurry KR et al. developed transgenic swine expressing human DAF and CD59 proteins, and achieved prolongation of survival and reduced vascular injury of swine hearts transplanted into non-human primates (McCurry K R et al., 1995 **272**). These findings were further enhanced by reports that cardiac allografts lacking DAF were vulnerable to HAR in the presence of low anti-α1.3Gal antibodies against wild type hearts (Shimizu I et al., 2006 **273**). Interfering with complement not only protects from aggressive humoral responses but has also provided evidence about the poorly understood mechanism of accommodation. In a xenotransplant model where hamster hearts were grafted into rat recipients, inhibition of MAC not only prevented HAR but also achieved a state of accommodation of these grafts (Suhr BD et al., 2007 **274**).

Allotransplantation in pre-sensitised rodents has done much to increase the understanding of the potential for complement regulation in HAR of donor hearts and

kidneys. Particularly promising results have been generated in models of both rats and mice that were pre-sensitised with donor skin (a powerful method for inducing alloantibody responses) prior to organ transplantation. This revealed that inhibition of the terminal complement components can afford protection of the transplanted organ against acute and hyperacute rejection in both rats (Brauer RB et al., 1995 **275**) and mice (Wang H et al., 2007 **276**; Rother RP et al., 2008 **277**), confirming data from xenotransplant models. So, rodent allotransplant models reproducing some of the most aggressive types of AMR are amenable to intervention using modulators of the innate immune response in the presence of alloantibody.

These results set the stage and direction for the research described in this thesis. The aim was to establish an appropriate rodent model in which to study HAR and determine the effects of localised inhibition of the complement (and coagulation) cascades. Whereas previous studies have used systemic anti-complement therapy often given in repeated doses, the aim was to avoid systemic or sustained disruption of the innate immune response, in order to minimize dangerous side effects including the risk of infection.

1.15 The Development of Localised Complement and Coagulation Regulators for Therapeutic Use

1.15.1 Membrane-Targeted Complement Regulator Mirococept (APT070)

Mirococept, also known as APT070, is a derivative of the natural regulator complement receptor 1 (CR1; CD35). CR1 is expressed on the surface of a variety of cells including peripheral blood cells (erythrocytes and lymphocytes) and plays a key role in immune clearance and complement inhibition at the cell surface (Roozendaal R et al., 2007 **278**). CR1 is a 160-250 kDa glycoprotein which is composed of numerous short consensus repeat domains (SCRs), a transmembrane and a cytoplasmic domain (Ahearn JM et al., 1989 **279**). The most common allotype, the A-allotype, is composed of 30 SCRs and within this structure there is an internal homology with long homologous repeats (LHR) every seven SCRs (Klickstein LB et al., 1987 **280**) (Figure 1.10A).

An alternative, recombinant form of CR1 which is soluble in plasma (Yoon SH et al., 1985 **281**) has been produced by gene transfection of Chinese Hamster Ovary (CHO)

cells (Weisman HF et al., 1990 **282**). The soluble human CR1 (sCR1) has been used successfully in a non-sensitised rat kidney transplant model of allograft rejection with limited mismatch between donor and recipient. In this model systemic administration of sCR1 reduced the level of vascular rejection (Pratt JR et al., 1996 **283**) and influenced primary alloantibody responses (Pratt JR et al., 1997 **284**).

Mirococept is produced by expression in *Escherichia Coli* with the potential for large amounts of protein to be generated (Dodd I et al., 1995 **285**). Mirococept, is not a traditional biopharmaceutical product, because it is not fully encoded by the gene with a synthetic tail incorporated into the agent by post-translational modification. Mirococept is a CR1 based chemically modified recombinant protein which is significantly smaller (24 kDa), than the native protein. It comprises the first three N-terminal short consensus repeats (SCRs) of human CR1 and a synthetic membrane-binding tail. More specifically, sCR1-3 of LHR-A is attached to a C-terminal cysteine, to enable it to be chemically linked to the membrane targeted tail. The design of the tail is such that it offers a two-site interaction with the phospholipid bilayer. The membrane inserting myristoyl forms hydrophobic interactions whereas a positively charged peptide sequence allows electrostatic interactions to occur with the negatively charged phospholipid bilayer (Figure 1.10B). As a result, haemolytic assays have shown that this cytotopic therapeutic has a 100-fold increased potency compared to the CR1 parent inhibitor (Smith RAG, 2002 **286**).

A B

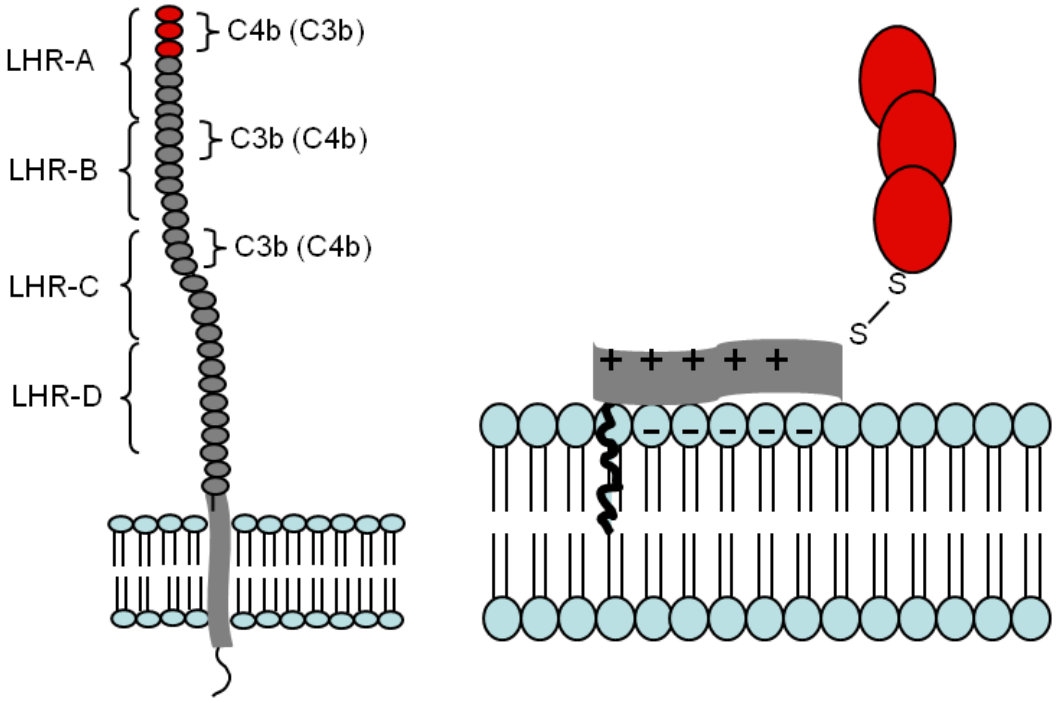


Figure 1.10A: Structure of human CR1, B: Structure of Mirococept (Smith RAG, KCL)

There are distinct advantages to this cell membrane targeted therapeutic technology, especially when trying to inhibit inflammation which is organ specific. It enables the interference of fluid-phase complement activation to be avoided (Mollnes TE et al., 2006 **287**). This approach targets complement inhibition by introducing the therapeutic regulator into the donor organ where it remains bound and so does not put the recipient at risk of infections. The fact that Mirococept is a lot smaller than CR1 allows it to bind more effectively to the cell surface with a dissociation constant that is lower than the native molecule's (Inflazyme Pharmaceuticals, research report 2005).

Mirococept has been extensively used and found to have cytoprotective effects in various animal models. The first *in vivo* study of Mirococept was in a rat model of acute vascular shock in which 80% of rats injected, intravenously, with a rabbit antibody,

died. Treated rats showed dose dependent effects of Mirococept ultimately with 100% survival (Smith RAG, 2002 **286**). Other studies investigating the benefits of Mirococept therapy have included models of experimental rheumatoid arthritis in the rat (Linton SM et al., 2000 **288**), and myocardial infarction in pigs (Banz Y et al., 2007 **289**). Most importantly, Mirococept has been found to be effective in improving outcome in renal transplantation. In a mild model of rat transplantation between Fischer and Lewis rats, Mirococept prolonged survival with a marked reduction in leukocyte infiltration (Pratt JR et al., 2003 **290**). Finally, in a study where rat kidney isografts were used, intra-renal delivery of Mirococept protected the donor kidney from fatal ischaemia/reperfusion injury (Patel H et al., 2006 **291**). In humans, Mirococept has successfully completed Phase 1 (safety/tolerance trials) (study report ME0579, KCL, unpublished) and Phase 2a pilot (Smith R/Sacks S unpublished data) studies. It is imminently to be used in a multicentre clinical trial in which the donor kidney grafts are treated with the reagent prior to transplantation to try and reduce ischaemia/reperfusion injury and delayed graft function (DGF) in transplant patients.

1.15.2 Membrane-Targeted Coagulation Regulators (PTL006, Thrombalexin-PTL004)

Using the same principle applied for the production of Mirococept (previously described) and other targeted therapeutics, a series of membrane-associated anticoagulants have been generated from a minimised form of hirudin. Hirulog-like peptide (HLL), a specific thrombin inhibitor, is isolated from the salivary glands of European medicinal leeches *Hirudo medicinalis* (Fenton JW 2nd et al., 1991 **292**). Thrombin is generated from prothrombin and it has multiple and diverse roles in coagulation, inflammation and wound healing. The product of activation is α-thrombin which has high fibrinogen clotting activity as opposed to β- and γ-thrombins (Fenton JW 2nd et al., 1991 **293**). Hirudin is a direct bivalent thrombin inhibitor and interacts with α-thrombin at two sites: the main classical active site (the catalytic site) and a unique, remote exosite (recognition site) which functions independently to the active site. Thrombin has multiple recognition domains, however, the structure of hirudin complements that of the fibrinogen recognition site (FRS) (De Cristofano R et al., 2003 **294**). The FRS is recognized by a variety of protein ligands including fibrinogen, PAR-1 on a variety of cells, heparin cofactor II, thrombomodulin (TM) and coagulation

factors V, VIII and XIII (De Cristofano R et al., 2003 **294**). The recognition site is critical for the enzyme's efficient recognition and interaction with substrates and inhibitors and thereby pivotal to thrombin's role in a variety of biological responses (Bode W et al., 1997 **295**).

Following this multi-point ligand structure, the therapeutics PTL006 and PTL004 were generated as part of a series of cytotopic anticoagulants prepared by solid phase synthesis. They vary in the nature of their membrane binding tail as will be discussed. The HLL peptide has been C-terminally coupled to a membrane binding tail to create a conjugate. PTL006 has a molecular weight of 5.3 kDa and it binds the cell membrane phosholipid bilayer via a synthetic tail containing a polyethylene glycol (PEG)-modified phospholipid (Figure 1.11). Two fatty acyl groups are also present to ensure stable insertion into the phospholipid bilayer. Binding of PTL006 to the cell surface relies on hydrophobic interactions between the tail and phospholipid bilayer

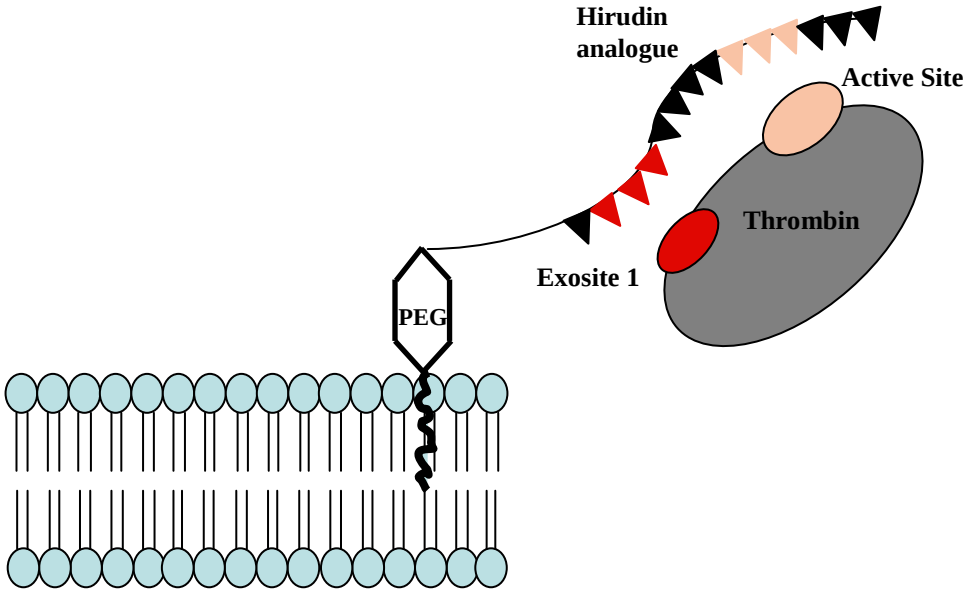


Figure 1.11: Structure of PTL006

Thrombalexin (PTL004), with a molecular weight of 4.6kDa, is composed of the HLL peptide with a disulphide link to a bis-myristoyl tail (Figure 1.12). The tail participates in both electrostatic and hydrophobic interactions with the cell membrane in a similar manner to Mirococept. The extra myristoyl group potentially results in a stronger interaction with the bilayer increasing stability at the cell surface.

Both of these novel therapeutic cytotopic (modified proteins hich bind cell surfaces) reagents have shown great promise *in vitro*. PTL006 was successfully able to incorporate into the cell surface of endothelial cells and at the same time inhibit TNF-α-induced TF expression. These cells effectively lost their procoagulant ability (Dorling A, unpublished data). Furthermore, in a cellular inhibition assay it was found that when red blood cells coated with either of the derivatives of tailed HLL, not only was thrombin captured from solution but also its activity was inhibited (Melchionna T, unpublished data).

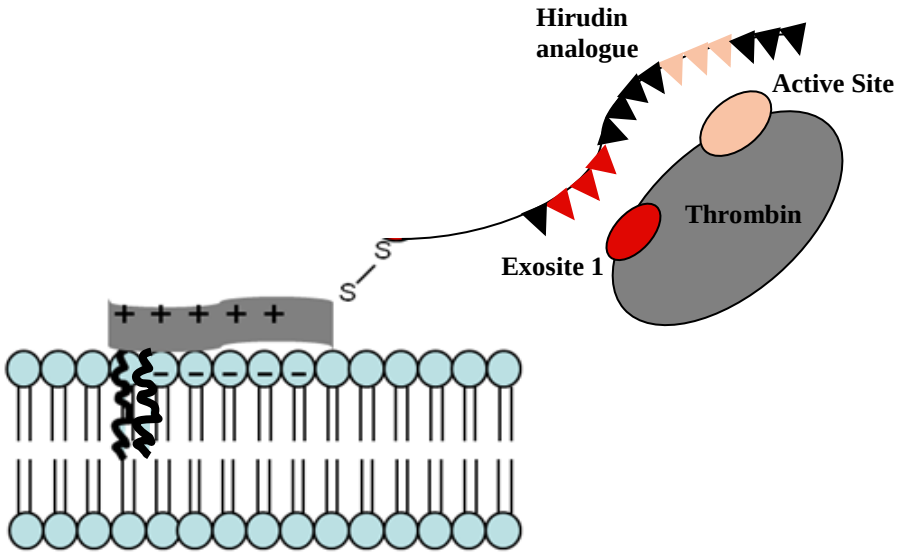


Figure 1.12: Structure of PTL004

These data demonstrate a potential for blocking thrombotic activity in transplantation cases where coagulopathy is associated with graft rejection. This thesis describes the first study where both PTL006 and PTL004 are tested in an *in vivo* model of rat kidney transplantation.

1.16 Hypothesis

Endothelial cell protection afforded by anchored therapeutic complement and/or coagulation regulators introduced into the donor kidney circulation will ameliorate graft loss associated with HAR. This will provide useful insight into the relative contribution of the complement and coagulation pathways in HAR. The data generated by this work will facilitate the transfer of the named inhibitors for the first studies in man. Hopefully this will ultimately contribute to better renal transplant outcomes in pre-sensitised patients.

1.17 Aims

The aims of my study were as follows:

Chapter 3: To establish a rat renal allograft model of hyperimmune antibody-mediated rejection using MHC class I and II disparate donors and recipients.

Chapter 4: To confirm both Mirococept inhibitory function *in vitro* and membrane binding ability *in vivo.* To test the ability of this membrane-targeted complement inhibitor to prevent rapid graft rejection in pre-sensitised recipients.

Chapter 5: To investigate the *in vivo* activities of PTL006. More specifically, to determine the optimal dose of PTL006 that achieves maximal anti-coagulant activity, with minimal complications in the rat model. Confirm the binding of PTL006 to cell membranes *in vivo*. To investigate the synergistic effect of the membrane-targeted complement (Mirococept) and coagulation regulator (PTL006) to prevent HAR in pre-sensitised recipients

Chapter 6: To test the ability of the membrane-targeted coagulation regulator PTL004 (Thrombalexin), to prevent rapid graft rejection in pre-sensitised recipients. To test the additive effect of standard T cell immunosuppression (Cyclosporin/Rapamycin) with Thrombalexin on graft survival.

Chapter 2 – Materials and Methods

2.1 *In vivo* Experimental Procedures

2.1.1 Animals

All animals were purchased from Charles River (Kent, UK). Animals utilized in this project were inbred male DA ($RT1^a$) rats used as skin donors, kidney donors and recipients. Inbred male Lewis ($RT1^l$) rats were used only as transplant recipients. Third party controls were Sprague Dawley (SD, outbred). Due to differences in size, the naturally larger Lewis rats were ordered as young as possible (150 g) at the initiation of the pre-sensitisation period. DA rats were ordered at simultaneously at 200 g to maximize their weight at the time of kidney transplantation. Animals had free access to standard rat chow and water. All procedures were carried out in accordance with a Home Office license for animal experimentation.

2.1.2 Pre-sensitisation of recipient Lewis rats by DA tail skin Transplantation

Prior to transplantation, Lewis recipients were pre-sensitised by sequential grafting of three full thickness tail skin segments (~2 cm^2) from DA donors. All rats were anaesthetised by inhalation of oxygen and isoflurane (Nicholas Piramar Ltd, distributed by IVAX Pharmaceuticals). The donor tail skin segments were placed in saline-soaked gauze at 4ºC while the recipients were prepared. A same size skin section was removed from the dorsal side of the recipient. The skin graft was held in place by applying a skin adhesive gel (Germolene New Skin, Boots), which was covered with a paraffin-coated dressing **(**Jelonet, Smith&Nephew, UK**)** and bandaged for seven days, after which the bandages were removed. Rejection was monitored by observation and defined as total necrosis of the tissue. The first two skin transplants were two weeks apart which was followed by a four week rest period. The third and final skin transplant was then given and a week later, these Lewis recipients received an allogeneic DA kidney graft.

2.1.3 Therapeutic Agents

The following reagents were kindly provided by Dr Richard Smith (KCL, London):

APT070 (Mirococept): This complement inhibitor comprises of a recombinant SCR1-3 of a human CR1 modified with a cytotopic peptide APT542. Mirococept was prepared on a large scale under cGMP conditions for clinical trials by Avecia Ltd (now Fujifilm Diosynth), Billingham, UK.

APT542: The tail of APT070 is made up of a N-(myristoyl)-GSSKSPSKKKKKKPGD-(S-2-thiopyridyl)-Cys-carboxamide sequence.

APT154: This denotes soluble untagged SCR1-3 which retains inhibitory activity in solution.

PTL006: This coagulation inhibitor is a conjugate of the 23 aminoacid hirudin-derived peptide (Hirulog/HLL).
H-(D) FPRPGGGGDGDFEEIPEEYLGGC-amide with a membrane-inserting tail of DSPE-PEG (2000) PDP [1.2distearoyl-sn-glycero-3-phosphoethanolamine-N-PDP (polyethelene glycol)-2000] (Avanti Pharmaceuticals).

PTL006-FAM: Inactive form of PTL006, labeled at the N-terminus of the peptide with FAM (carboxyfluorescein).

PTL004 (Thrombalexin): Coagulation inhibitor comprising of a 23 amino acid hirudin-derived peptide (Hirulog/HLL) linked to the membrane-localizing tail (bis-myristoyl).
Structure: H-(D) FPRPGGGGDGDFEEIPEEYLGGC-amide with N-(bis-myristoyl)-KSSKSPSKKDDKKPGD-(S-2-thiopyridyl)-Cys-carboxamide.
Peptides were prepared by Cambridge Research Biochemicals, Billingham, UK.

All reagents were perfused in Soltran kidney perfusion solution (Baxter Healthcare Ltd). This composed of:

Potassium Citrate	8.6 g
Sodium Citrate	8.2 g
Mannitol	33.8 g
Magnesium Sulphate	10.0 g

2.1.4 Renal Transplantation

The technique used was adapted from a previously published method (Fabre J et al., 1971 **296**) and is life-sustaining when performed between syngeneic or allogeneic donors and recipients, due to nephrectomy of the native kidneys. All rats were anaesthetised by inhalation of oxygen and isoflurane (Nicholas Piramar Ltd, distributed by IVAX Pharmaceuticals).

2.1.5 Donor Preparation

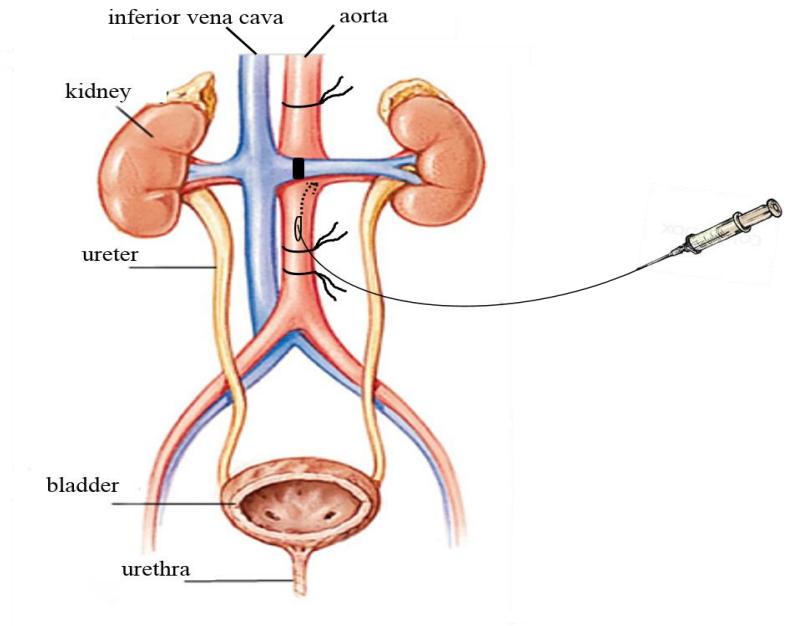
Donors and recipients were prepared simultaneously so that there was no cold ischaemia time. To enable this to happen, all donors were prepared by Dr Conrad Farrar as I prepared the recipients and subsequently carried out the perfusion and renal transplant procedure. DA rats were used as kidney donors. For the donor preparation, a warm heat mat was placed underneath the animal to maintain a constant body temperature. A midline laparotomy was performed isolating the left kidney, the renal vein and artery, aorta and inferior vena cava (IVC). All arterial branches from the aorta and artery were tied off using 7/0 braided silk wax sutures (Pearsalls Ltd, UK). Ligatures were also placed above and below the renal pedicle. This ensured that all reagents were delivered directly to the kidney and could not go elsewhere.

2.1.6 *Ex vivo* Perfusion of Therapeutic Agents prior to Transplantation

The protocol for the cytotopic delivery of therapeutic reagents is well established in our department and has been successfully used in rat models of allotransplantation (Pratt JR et al., 2003 **290**) and ischaemia/reperfusion injury (Patel H et al., 2006 **291**). A portex catheter (Smiths Medical, Kent, UK) attached to a three-way tap, was inserted in the aorta and intrarenal delivery of 5ml of Soltran perfusion solution (Baxter Healthcare Ltd, UK), with or without therapeutic reagent, was performed. The perfusion was over 5 minutes, at 1ml/min. This protocol was used for the delivery of Soltran, complement inhibitor APT070 and untagged inhibitor APT154 into the kidney. The procedure was modified to include two extra steps when delivering coagulation inhibitor PTL006, PTL006-FAM, and PTL004. These were: 1) a ten minute wait period post-perfusion. During this time the heat mat was switched off whilst the kidney graft was constantly bathed in with ice cold saline. Subsequently there was a wash through step: 2) A second perfusion of the kidney with 5ml of Soltran alone at a rate of 1 ml/min. The kidney was

then excised and immediately transplanted into the recipient. A schematic presentation of the procedure is shown in figure 2.1.

Figure 2.1: Intragraft delivery of Soltran perfusion solution



DA Donor Kidney

↓

Intragraft Delivery of Soltran Perfusion Solution

↓

Hyperimmune Lewis Recipients

Schematic of protocol used for the perfusion of the vehicle Soltran solution. The inferior vena cava and aorta were isolated and a portex catheter attached to a syringe, containing 5 ml of Soltran, was inserted into the aorta. During this procedure, the preservation solution was delivered through the renal artery directly into the kidney at a rate of 1 ml/min, for a total 5 minutes.

2.1.7 Recipient Preparation

For the recipient preparation, a heat mat was placed underneath the animal to maintain constant body temperature. A midline laparotomy was performed followed by removal of the left kidney. Microaneurysm clips (Johnson & Johnson, UK) were used to clamp the renal vein and artery. The donor kidney was transplanted orthotopically in an end to end anastomosis with the renal vein, artery and ureter using 10/0 sutures (Bear Medic Corp. Japan). Following a warm ischaemic time of approximately 25 minutes, the clips were removed to restore blood flow to the transplanted kidney. Finally, a second native nephrectomy was carried out to ensure that the survival of the recipient was dependent on the transplanted kidney alone. The end point of graft survival was defined by death of the recipient or when the animal had to be culled due to signs of distress.

As predicted, technical failures accounted for approximately 20% of the transplants performed. They were attributed to either failed perfusion of the reagents (thus not successful intrarenal delivery of therapeutic reagents) or complications during or after anastomosis. Surgical complications were mainly due to mismatch in the size of the donor (smaller) and recipient (larger) arteries. Furthermore, approximately 30% of recipient rats (Lewis) had two renal arteries thus complicating the arterial anastomosis between the graft and recipient. Post-operative complications included abdominal urine leakage due to ureteric obstruction resulting from inconsistency in size of the donor and recipient ureters.

2.1.8 Administration of Cyclosporine A and Rapamycin

Treatment of rats with Cyclosporine A (Sandimmune®, Sandoz Pharmaceuticals, Surrey, UK) commenced 2 days prior to renal transplantation at a dose of 10mg/kg/day intraperitoneally. Rapamycin (LC Laboratories, Woburn, MA, USA) was administered on the day of transplantation at a dose of 1.5mg/kg/day intraperitoneally. Both treatments were continued daily until the end of the experiment.

2.1.9 Collection of Blood and Extraction of Serum

Pre- and post- renal transplantation blood samples were collected by tail vein sampling. During the pre-sensitisation period, weekly samples of ~20 µl of blood were taken from the tail vein of the Lewis rats into 1.5 ml eppendorf tubes. For serum preparation, this whole blood was then centrifuged for 10 minutes at 14000 rpm. The serum was collected and the spin repeated. Sera were stored at -70 °C until required for analysis. Serum taken prior to renal transplantation was used to assess alloantibody titres, during the sensitisation period. Post-renal transplantation, Lewis rats were bled daily to measure BUN, thereby monitoring renal function until death of the recipients. Terminal blood samples were collected by cardiac puncture.

2.1.10 Rat Splenocyte Extraction

Rat splenocytes were obtained by disruption of the newly harvested spleen by pressing through a 70 µm sieve over a 50 ml falcon tube containing ~20ml of sterile PBS (Fischer Scientific, UK). The cells were spun down by centrifugation and resuspended in 10 ml red blood cell (rbc) lysis buffer for 5 minutes on ice. Lysis was terminated by adding PBS/2%/ FCS (Sigma-Aldrich, UK) up to 50 ml. The remaining PBMC were spun down, resuspended in 20 ml PBS/2%/FCS and then re-spun. The final washed cell pellet was resuspended in 10 ml of sterile PBS/2%/FCS and viable cells were counted using a haemocytometer (Neubauer Improved, Germany) with trypan blue viability dye (Sigma, UK). The cells were either analysed further or frozen.

Red blood cell (rbc) lysis buffer

4.17 g of NH_4Cl

0.0185 g of EDTA

0.5 g of $NaHCO_3$ all dissolved in 500 ml of deionised H_2O.

The solution was autoclaved and stored at 4 ºC until required.

2.1.11 Freezing Splenocytes for Storage

Splenocytes were collected by centrifugation and resuspended, drop by drop in sterile freezing mix comprising 10% DMSO (Sigma-Aldrich, UK) and 90% FCS. The cells were then aliquoted into cryogenic vials (Nalgene, UK), stored in a freezing container (Mr Frosty, Thermoscientific) overnight at -70 °C before long term storage in liquid nitrogen.

2.1.12 Thawing of Splenocytes

Under sterile conditions, cells were transferred from the cryogenic vials in 15 ml tubes containing 10 ml RPMI/ 10% FCS. Cells were spun down at 1600 rpm for 5 minutes. The cell pellet was resuspended in 10 ml RPMI/ 10% FCS and counted before used, as previously described.

2.2 *In Vitro* Experimental Procedures

2.2.1 Detection of Alloreactive IgG

During the sensitisation period, blood was acquired weekly by tail vein bleeding for serum analysis of alloantibodies and BUN. Serum was prepared as previously described (2.1.9) and stored at -70 ºC until required for analysis. Donor DA or third party SD splenocytes were aliquoted ($1x10^6$/well) into wells of 96 well v-bottom plates and spun down by centrifugation at 1600 rpm for 5 minutes. After flicking off the supernatant the cells were pre-blocked by resuspension in 50µl PBS/2% BSA containing 20% normal

mouse serum. The cells were left on ice for 20 minutes. For gating of T-cells, mouse anti-rat CD3-PE (clone G4.18, Ebioscience Ltd) monoclonal antibody was added and incubated at 4 ºC for 20 minutes. Cells were then washed and resuspended in PBS/2% BSA into which serum was added to give final dilutions of 1:10, 1:50, 1:250, 1:1250 and 1:6250. Cells were incubated on ice for 20 minutes. Splenocytes were washed twice with PBS/2% BSA as before and then incubated with mouse polyclonal anti-rat IgG-FITC (H+L, eBioscience Ltd,) for 20 minutes on ice. Cells were then washed twice and fixed in 2% paraformaldehyde before being analysed on a FACScan flow cytometer (BD).

2.2.2 Assessment of Renal Function by measuring Blood Urea Nitrogen (BUN)

Tail vein blood was taken daily from the rats post transplantation and serum prepared as previously described (2.1.9). For BUN analysis 5 µl of serum was added to 500 µl of Infinity Urea (BUN Infinity, Fisher Scientific, Loughborough, UK) and two readings, at 30 seconds and then 90 seconds, were measured on a spectrophotometer (Beckman Coulter, Oxford, UK) after standards were determined using a urea/glucose standard (Thermotrace, Australia). The readout was absorbance at 340 nm and BUN values were calculated according to the manufacturer's instructions.

2.2.3 Harvesting Tissue for analysis of Renal Pathology

At appropriate time points kidneys were removed and preserved in 4% paraformaldehyde (Sigma-Aldrich, UK). They were then processed overnight using a Tissue Tek processor before embedding in paraffin. Tissue sections (2µm) were then cut using a sledge microtome (Leica) and floated in a 44 ºC water bath. Sections were transferred onto super premium microscope slides (VWR International, Leicestershire, UK) and allowed to air-dry. Prior to staining, sections were placed in a 65 ºC oven for 1 hour to melt the wax. The sections were then de-waxed by immersion in xylene for 5 minutes and then re-hydrated in water through graded alcohols [100% (2 changes); 90% (2 minutes)].

2.2.3.1 Histological Procedures

2.2.3.2 Haematoxylin & Eosin (H&E) Staining

Re-hydrated tissue sections were immersed in haematoxylin (RA Lamb, UK) for 7 minutes followed by a 15-20 minute wash with running water. Sections were then dipped in eosin (RA Lamb, UK) for 5 minutes and rinsed briefly in water. They were then dehydrated by immersing through increasing concentrations of alcohol (90%, 100%). Finally, sections were cleared in xylene and mounted in DPX (dibutyl phthalate containing xylene) (VWR, UK). Haemotoxylin stains the nuclei of cells dark blue and the cytoplasm is stained pink by eosin.

2.2.3.3 Periodic Acid Schiff (PAS)

Rehydrated tissue sections were placed in 1% periodic acid (Sigma) for 10 minutes followed by a 5 minute wash with distilled water. Sections were then placed on a rack and Feulgen's Schiffs (RA Lamb Ltd) was dropped onto each section and left for 10 minutes. After washing in running tap water for 5 minutes, sections were immersed in haematoxylin for a further 3 minutes. Following a final wash in running tap water for 15-20 minutes, sections were dehydrated as previously described, then mounted in DPX. PAS stains periodic acid positive tissue pink. Haemotoxylin stains the nuclei of cells dark blue.

2.2.3.4 Martius Scarlet Blue (MSB)

De-waxed and rehydrated kidney tissue sections were placed in a 3% potassium dichromate/10% acid alcohol (Sigma) solution for 5 minutes followed by a short rinse with running water. Iron alum was then added for 5 minutes followed by a 5 minute immersion in haematoxylin. The sections were then washed with running tap water for 10-20 minutes, rinsed briefly with 95% ethanol and immediately stained with martius yellow for 5 minutes. Following a brief rinse with distilled water the sections were stained with brilliant scarlet crystal for 20 minutes. Finally, using a pastette, soluble blue was dropped carefully onto each section and left for precisely 1 minute. The sections were then blotted lightly on tissue paper, then dehydrated, cleared in xylene and mounted in DPX as previously described. MSB stains fibrin scarlet, the muscle pink, the RBC's yellow and collagen blue.

3% potassium dichromate/ 10% acid alcohol buffer

3 parts of 3% potassium dichromate and 1 part 10% hydrochloric acid in 95% alcohol

Iron alum buffer

15 g ferric ammonium sulphate dissolved in 300 ml dH_2O

1.5 g of celestine blue was added to the solution and boiled for 3 minutes

Once cool, the solution was filtered by pouring through a pre-cleared filter paper funnel

42 ml of glycerol was added.

0.5% martius yellow buffer

1.5 g martius yellow and 6 g phosphotungstic acid in 300 ml of 95% EtOH

1% brilliant crystal scarlet buffer

3 g of brilliant crystal scarlet in 392.5 ml dH_2O and 7.5 ml acetic acid

0.5% soluble blue buffer

1.5 g of aniline blue and 3 ml acetic acid in 297 ml dH_2O

All solutions were stored at room temperature until required

Features of rejection were evaluated and assessed by Dr Catherine Horsfield (Consultant Histopathologist, St Thomas' Hospital, London, UK) in accordance with Banff criteria.

2.2.4 Haemolytic Assay for the Activation of the Classical Complement Pathway and Inhibition by APT070 (Mirococept)

This assay was used to determine the functional inhibition of the classical complement pathway by the inhibitor Mirococept. Antibody-sensitised sheep erythrocytes (EZ complement cells, Diamedix Labmedics, Oxford**)** were aliquoted into the wells of a 96-well v-bottom plate (100 µl/well) and incubated with rat serum (50 µl) at a 1:160 dilution in CPHAD buffer. This caused maximal haemolysis of the EZ cells. In other wells a titration of APT070 or non-tailed control APT154 was added at 125, 50, 20, 8, 3.2 1.28, and 0.512 nM to a total volume of 200 µl. Duplicate wells were prepared, and all dilutions were carried out in classical pathway haemolytic assay diluents buffer (CPHAD). A lid was put on the plate which was gently agitated to mix the contents before incubation for 1 hour at 37 ºC in an incubator. The plate was then centrifuged at 1600 rpm for 3 minutes. Finally, 150µl of supernatant was transferred into a replica 96-well flat-bottom plate and the absorbance was read at 405 nm using a microplate reader (Spectromax, UK).

The % haemolytic inhibition (% HI) was calculated using the following equation:

$$\% \text{ HI} = 1 - \left(\frac{t - A°}{Amax - A°}\right) x\ 100$$

where:

t = mean OD for a sample

A° = cells and buffer alone (spontaneous lysis)

Amax = cells and rat serum alone (maximum lysis)

Classical pathway haemolytic assay diluents buffer (CPHAD)

11.9g of HEPES (BDH)

4.4g of NaCl (BDH)

300ml of deionised H_2O in a 500 ml bottle.

Gelatin (1.5g) was then added (Sigma) and the solution was heated in SHORT bursts in microwave until the gelatin was dissolved.

pH was adjusted to 7.4 and the volume was made up to 500ml with dH_20

The solution was warmed to 37 °C, filtered into a new bottle and and stored at 4 °C

As required, an aliquot (50 ml) was heated to 37 °C and then maintained at RT for use

2.2.5 Tissue Collection for Frozen Sections

At appropriate time points grafted kidneys were removed and placed on cork disks (RA Lamb) in silver foil cups and coated with optimal cutting temperature (OCT) compound (RA Lamb) for snap freezing in liquid nitrogen. Tissue sections were cut (5μm) using a cryostat (Bright Instrument Ltd, Huntington, UK) and transferred onto multispot glass slides (Hendley-Essex, Loughton, UK). They were left to air dry overnight before being stained and analyzed or stored at -70 ºC in 50 ml falcon tubes for processing at a later date.

2.2.6 Immunofluorescence (IF)

2.2.6.1 C4d Staining

Frozen tissue sections on slides (as previously described) were fixed in ice cold acetone for 10 minutes, then left to air dry for 30 minutes. Sections were blocked with 20% rabbit serum (the species of origin of the secondary reagent) for an hour and after a brief wash (PBS), sheep anti-rat C4d antibody (courtesy of Dr Sarah DeFreitas) was added at a 1:80 dilution (PBS) for 90 minutes. After 3 washes in PBS, sections were incubated with rabbit anti-sheep IgG FITC (Santa Cruz, distributed by Insight Biotechnology, UK) at a 1:200 dilution for 75 minutes at RT. Three PBS washes followed before a final 5 minute wash was performed with water. The slides were then mounted in fluoromount

(Permafluor, Fisher Scientific, UK) and covered with glass cover slips for analysis using a fluorescence microscope (Olympus, UK).

2.2.6.2 APT070 (Mirococept) Staining

Detection of Mirococept in rat tissue followed the same immunofluorescent principles. Frozen tissue samples were fixed in ice cold acetone for 10 minutes, then left to dry for 30 minutes. Non-specific binding was prevented by blocking the sections with 20% goat serum for 1 hour at room temperature. After a brief wash in PBS, sections were incubated with mouse anti-human SCR1-3 (3E10, Adprotech) for 1 hour at a 1:100 dilution (PBS). Sections were then washed three times in PBS for 5 minutes, on a rotating platform. The secondary reagent goat anti-mouse IgG FITC (Jackson, UK) was applied to the sections at a 1:200 dilution for 30 minutes then washed three times as before. A final 5 minute wash was performed with water. The slides were then mounted in fluoromount (Permafluor, Fisher Scientific, UK) and covered with glass cover slips, before analysis using a fluorescence microscope.

2.2.6.3 C5b-9 (Membrane Attack Complex) Staining

Frozen tissue samples were fixed in ice cold acetone for 10 minutes, then left to dry for 30 minutes. Non-specific binding was prevented by blocking the sections with 10% FCS in PBS for 30 minutes followed by 20% goat serum in PBS at RT. After a brief wash in PBS, sections were incubated with mouse anti-rat C5b-9, (Hycult Biotech, UK) for 1 hour at a 1:50 dilution. Sections were then washed three times in PBS for 5 minutes, on a rotating platform. The secondary goat anti-mouse IgG FITC (Jackson, UK) was applied to the sections at a 1:200 dilution (PBS) for 30 minutes then washed three times as before. A final 5 minute wash was performed with water. The slides were then mounted in fluoromount (Permafluor, Fisher Scientific, UK) and covered with glass cover slips. All sections were stored in the dark at -4 ºC before analysis using a fluorescence microscope.

2.2.6.4 PTL006-FAM Detection

Snap frozen tissue sections were cut as previously described (4.20) and allowed to air dry for 30min before adding PermaFluor aqueous mounting medium to each section. The sections, kept in the dark and at room temperature, until being analysed using a fluorescence microscope.

2.3 Statistical Analyses

BUN values and haemolytic assay data were compared using the Student's *t* test. Survival data were analysed using the Kaplan Meier method (log-rank test) to show the significance in survival between animal groups. A difference was considered significant when $p<0.05$.

Chapter 3 - Establishing a Rat Hyperimmune Kidney Transplantation Model

3.1 Introduction

Transplants are powerful stimulants of alloantibody generation (Wehner J et al., 2007 **81**) and this is pivotal to graft rejection and loss. Previous studies in animal cardiac transplant models have demonstrated that alloantibodies (mainly of the IgG type) bind onto endothelial cells in the arteries, capillaries and veins post transplantation mediating injury to the cardiac vasculature (Minami K et al., 2006 **297**). This was further supported by similar findings by Qian Z et al. where rat cardiac recipients, pre-sensitised with blood transfusions, exhibited harmful vascular pathology associated with a strong IgG alloantibody responses (Qian Z et al., 2006 **298**). These experimental models clearly demonstrate the capacity of alloantibody to bind to large arterial endothelium and promote graft injury. More specifically, immune-mediated injury of the graft endothelium results in its activation and subsequent initiation of complement and coagulation cascades. These events contribute to damage and rejection of the graft.

The most severe effect of alloantibodies on a transplanted organ is hyperacute rejection. Hyperacute rejection is, by definition, immediate, occurring within minutes to hours post revascularization and leading to necrosis of the graft. This type of immunological reaction (caused by antibody binding to the donor organ), results in the disruption of the vasculature, indicated by the presence of complement and fibrin. Hyperacute rejection is uncommon, mainly due to cross match techniques used to identify the presence of antibodies in recipients against potential donors. However, hyperacute responses can occur following previous exposure of a recipient to HLA and/or ABO antigens, and may prevent successful kidney transplantation.

Animal models have aimed to investigate the role of alloantibodies in the rejection of transplanted organs. Most of this earlier research has focused on acute rejection rather than HAR, although the latter closely reflects the scenario of a sensitised patient in the clinic. HAR of vascularized transplants is difficult to achieve in rodent models even where there are high alloantibody titres. For this reason, HAR models have, in the past, been established through a xenotransplantation setting. Although in some respects xenoantibody responses are similar to the allogeneic response, they do not reflect the

situation in the clinic. Nonetheless, xenotransplantation models have furthered our understanding of how alloantibody and complement activation affect graft survival. Although rodent allotransplantation models offer significant knowledge on complement mechanisms, they have so far provided limited information regarding the thrombotic effects of vascular rejection, an area mainly covered by xenotransplantation models. The role of coagulation in acute rejection episodes is well recognised and intravascular thrombosis is considered a reliable marker of accelerated humoral rejection (Rose AG et al., 2000 **299**).

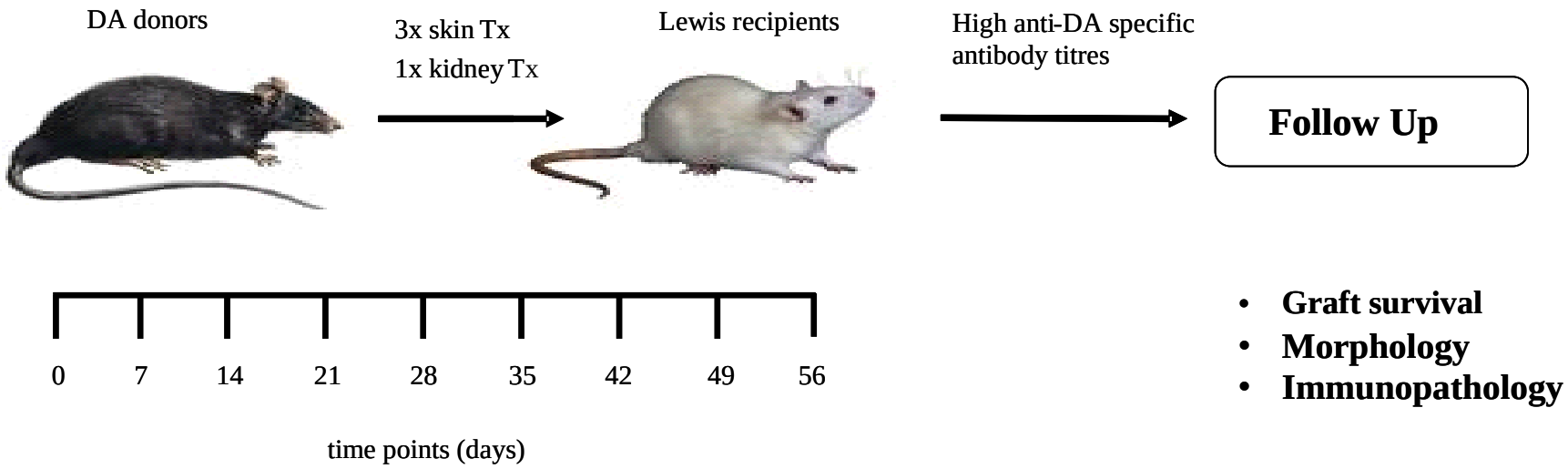
The aim of the research described in this chapter was to establish a rat kidney transplant model of HAR in which cell-protective therapeutic strategies could later be tested. The validity of this model was established by reference to the tempo and pathological changes associated with accelerated AMR, as described in an earlier publication (Truong LD et al., 2007 **56**).

3.2 Experimental Design

A model of hyperimmune renal allotransplantation was established using Dark Agouti (DA) rats as donors and Lewis rats as recipients. This high responder DA to Lewis rat strain combination is a well-established model of fatal acute kidney rejection. More specifically, transplantation of kidneys between these two rat strains has been shown to lead to acute destruction of the graft and death of the recipient with a median survival time of 6 days (Lewin E et al., 1994 **300**). The object of my experiments was to focus primarily on antibody mediated rejection. To enable this, the experimental design was adapted to incorporate a pre-sensitisation phase, as described in a previous study of rat cardiac allotransplantation. In this study, rats were sensitised with multiple donor strain tail skin grafts prior to heart transplantation (Brauer RB et al., 1995 **275**). In a similar fashion, the Lewis recipient rats in the model described for my work received a sequence of three DA full-thickness skin segments before being transplanted with a DA kidney. The rationale behind this design was to prime the immune response with two successive skin grafts and allow a gap for affinity maturation, to end up with a high affinity IgG response, which in clinical transplantation is associated with severe AMR. To boost the antibody titre prior to kidney transplantation, the animal received a third

skin graft from the same donor-strain. The DSA titre was determined at appropriate time-points after skin grafting. The read-out for the effect of pre-immunisation on subsequent renal transplantation in this model included: graft survival, graft morphology and immunopathology. A schematic of the hyperimmune kidney transplant model is shown in figure 3.1.

Figure 3.1:Schematic of hyperimmune rat renal transplantation model



Schematic of the protocol used to develop a hyperimmune rat renal transplantation model. Lewis recipients received DA strain tail skin transplants on days 0, 14 and 49. Renal allografts from the MHC I/II disparate DA donors were orthotopically transplanted into the hyper-sensitized recipients at day 56, at which point they were checked to have developed high anti-DA specific antibodies. All transplants were performed on sensitized recipients without any deliberate cold ischaemia. In the follow up period, graft survival, renal function and pathology were assessed to check for the presence of the characteristics establish of HAR.

3.3 Effect of Skin Grafting on Alloantibody Response prior to Kidney Transplantation

Recipient IgG responses measured by specific binding to donor (DA) are shown at different time points after skin transplantation (Figure 3.2). Donor-specific alloantibody shows a peak response at day 14 just prior to receiving a second skin graft. The dilution values performed after the third skin graft on day 49 confirm high titre alloantibody responses against donor splenocytes are present prior to kidney transplantation (Figure 3.3 A). Representative histograms of control experiments showing detection of background Lewis IgG against SD (third party) and DA serum against syngeneic targets (Figure 3.3 B and C).

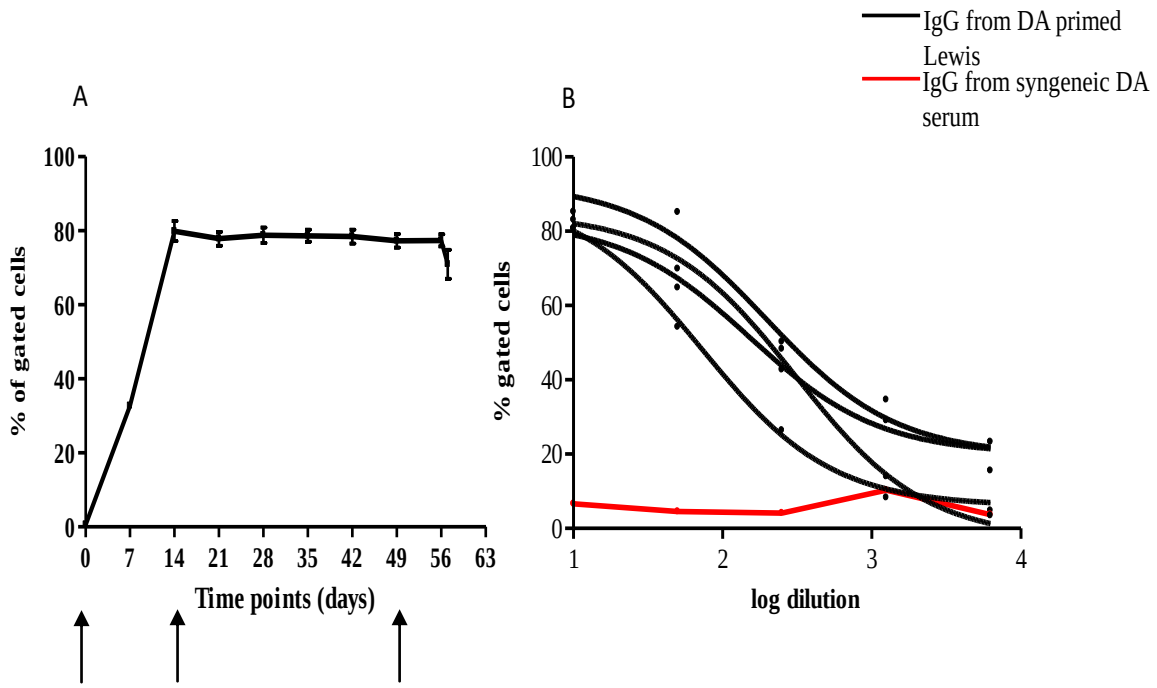


Figure 3.2 A: Sera from individual rats (n=4) was analysed by flow cytometry to detect a DA alloantibody response elicited by three successive DA skin allografts (at time points shown by arrows) measured weekly at 1:10 serum dilution. These are mean values of percent positive cells obtained from gating on the $CD3^+$ lymphocyte population. B: Titration curve of sera, from the same animals (n=4), analysed at day 56 to ensure high alloantibody titres pre-kidney transplantation. Donor specific IgG was detected on serially diluted sera (1:10, 1:50, 1:250, 1:1250 and 1:6250) and compared against syngeneic DA serum.

A

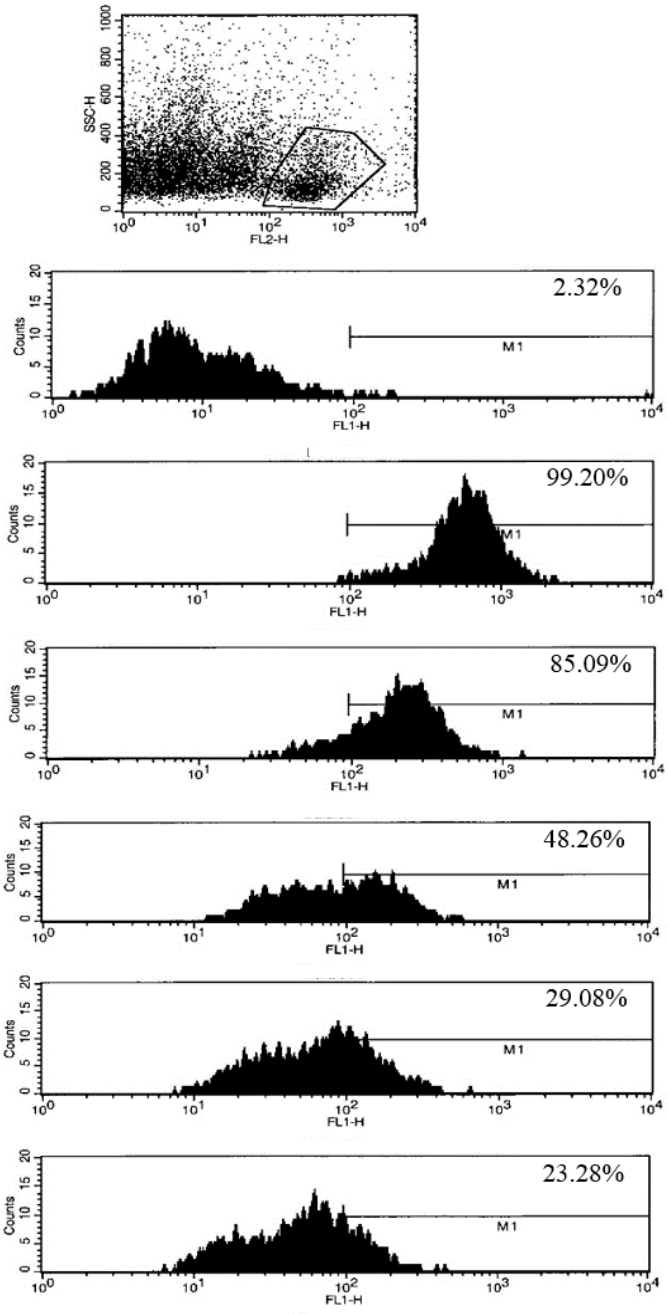


Figure 3.3 A: Donor specific IgG was detected in serially diluted serum (1:10, 1:50, 1:250, 1:1250 and 1:6250) from a sensitised Lewis recipient analysed at day 56 prior to kindey transplantation by FACS assay. High allo-antibody titres are shown. Top panel: isotype control (1:10).

B

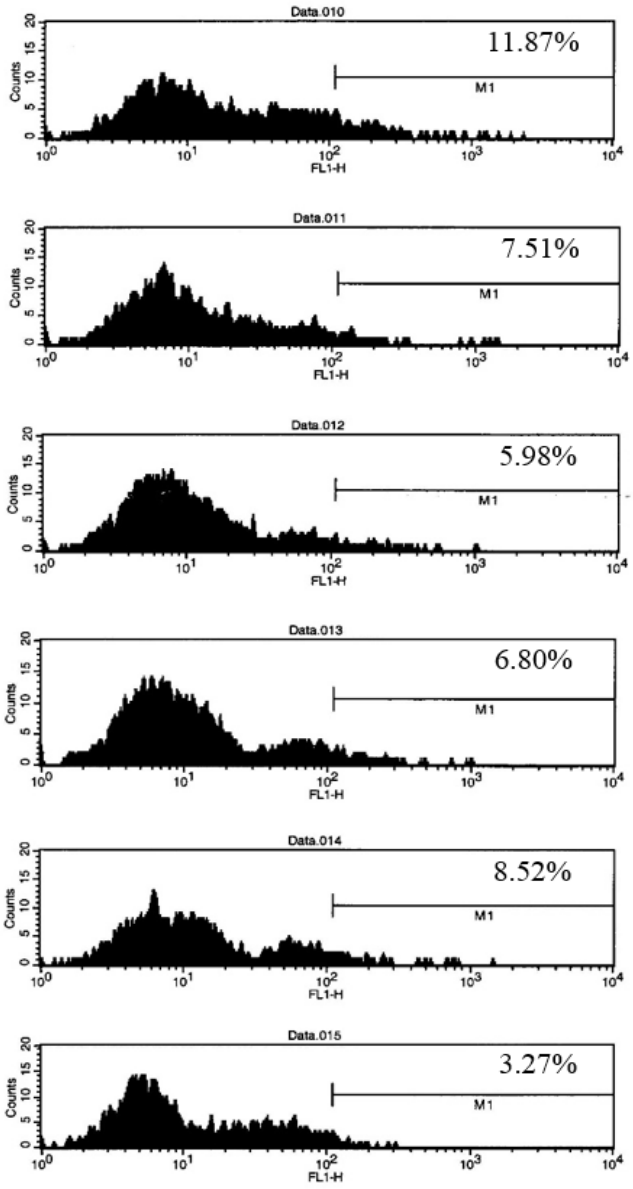


Figure 3.3 B: The absence of donor specific IgG in serially diluted serum (1:10, 1:50, 1:250, 1:1250 and 1:6250) from a DA syngeneic recipient analysed by FACS assay. Bottom panel: isotype control (1:10).

C

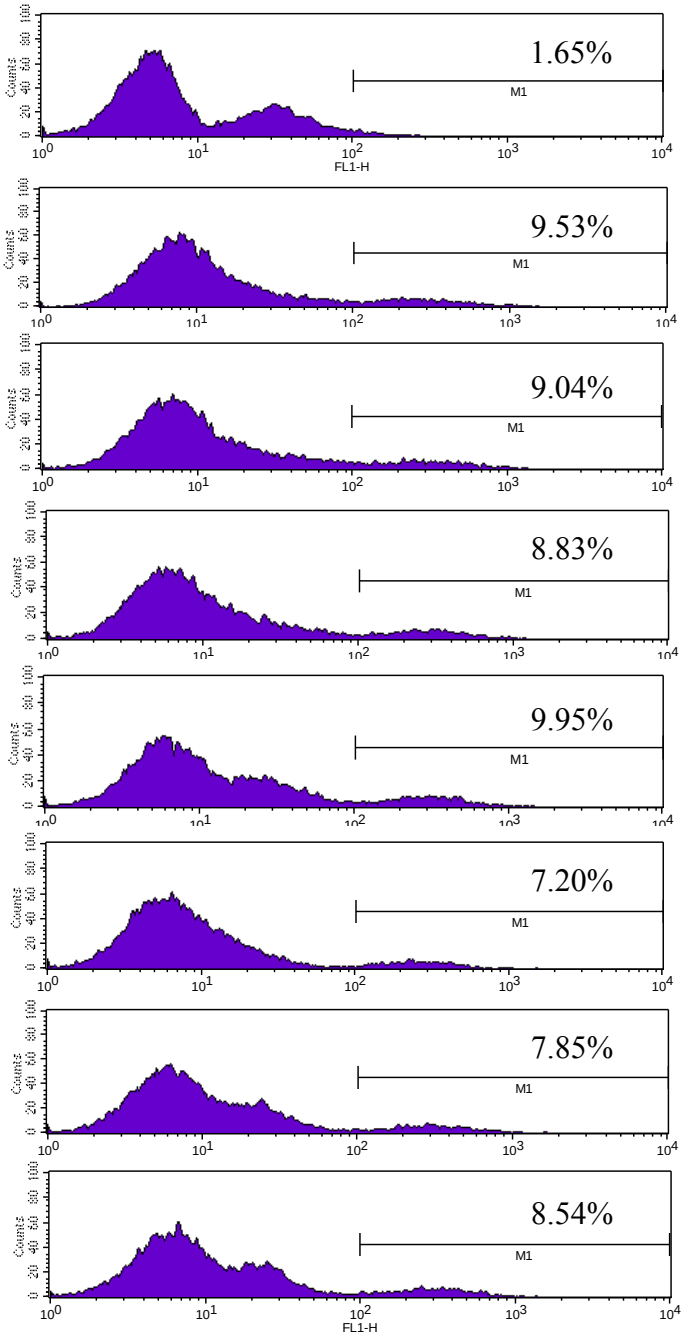


Figure 3.3 C: The absence of donor specific IgG in serially diluted serum (1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280) from a sentitised Lewis recipient against SD target splenocytes analysed by FACS assay. Top panel: isotype control (1:20).

3.4 Impact of Pre-existing Alloantibody on Tempo and Character of Renal Transplant Rejection

Pre-immunised rats (Lewis) showing high titres of DSA or control non-immunised rats (Lewis) were transplanted with kidneys from the same donor-strain (DA). Graft rejection was defined as high serum BUN, poor health or death of the animal. The results (Figure 3.4) show that graft loss is significantly shortened in the hyperimmune animals compared with non-sensitised controls. The mean survival times (MST) in the two groups were 1.8 days and 5.6 days respectively.

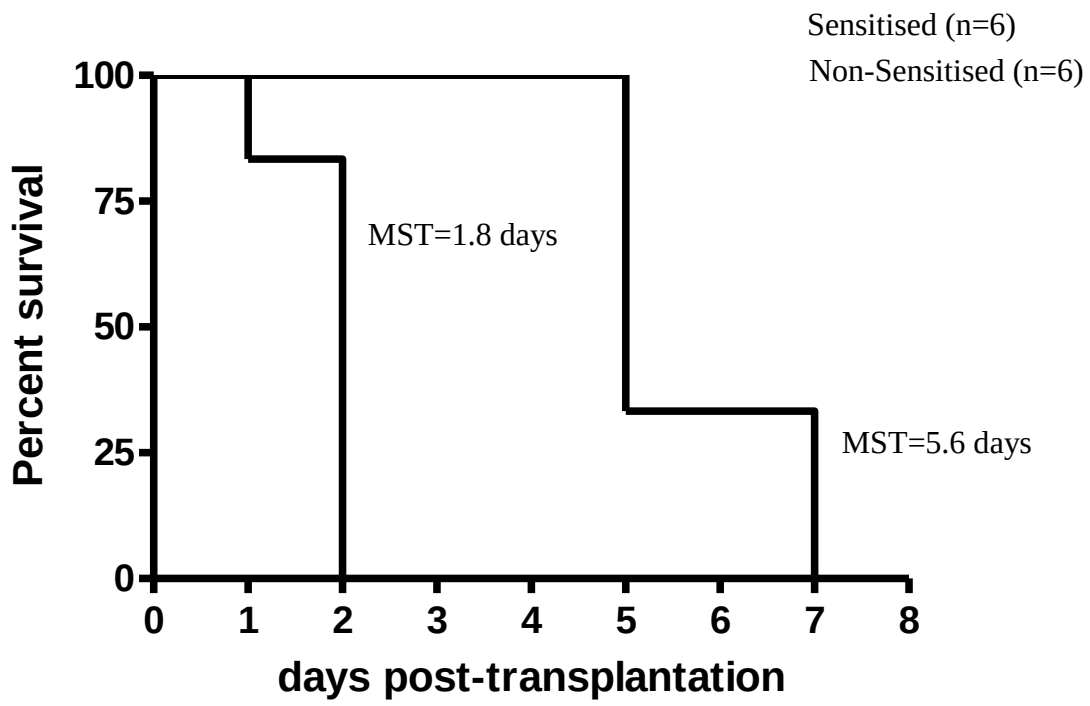


Figure 3.4: Kaplan-Meier survival curve showing graft survival in sensitised and non-sensitised recipient rats. Non-sensitised Lewis recipients rejected the DA donor kidneys in an acute manner. Lewis recipients, pre-sensitised with three DA skin grafts (n=6), showed accelerated rejection resulting in kidney loss.

3.5 Clinical parallels in Macroscopic Appearance of the Transplant

In clinical transplantation, HAR, due to preformed antibody results in immediate graft loss, usually within 24 hours after restoration of blood flow. This is because alloantibodies in the recipient's circulation instantly interact with the donor histocompatibility antigens on the donor vasculature thus initiating an acute vascular reaction which is ultimately responsible for necrosis of the graft. Progression of widespread capillary thrombosis results in a lack of perfusion. Typically, the kidney, within minutes, becomes flabby, mottled with purple patches and, in extreme cases, completely black.

In my model, hyper-sensitised Lewis rat recipients, having developed DA donor specific antibodies, were expected to elicit an instant immune reaction to the DA transplanted kidney minutes after revascularization. Figure 3.5 shows the observations following transplantation of a DA kidney transplanted into a sensitised Lewis recipient from the moment the miscrosurgical clips are released (to permit blood flow after the vascular anastamosis has been completed) to 10 minutes after the clamps had been released. In approximately 10% of the transplants carried out, the kidney had completely blackened by the time the bilateral nephrectomy was completed (Figure 3.5 E). This appearance is characteristic of diffuse thrombosis and tissue infarction, as described for HAR. In contrast, all syngeneic transplants survived the initial surgery, indicating that the surgical procedure was successful and HAR did not occur in the naïve recipients.

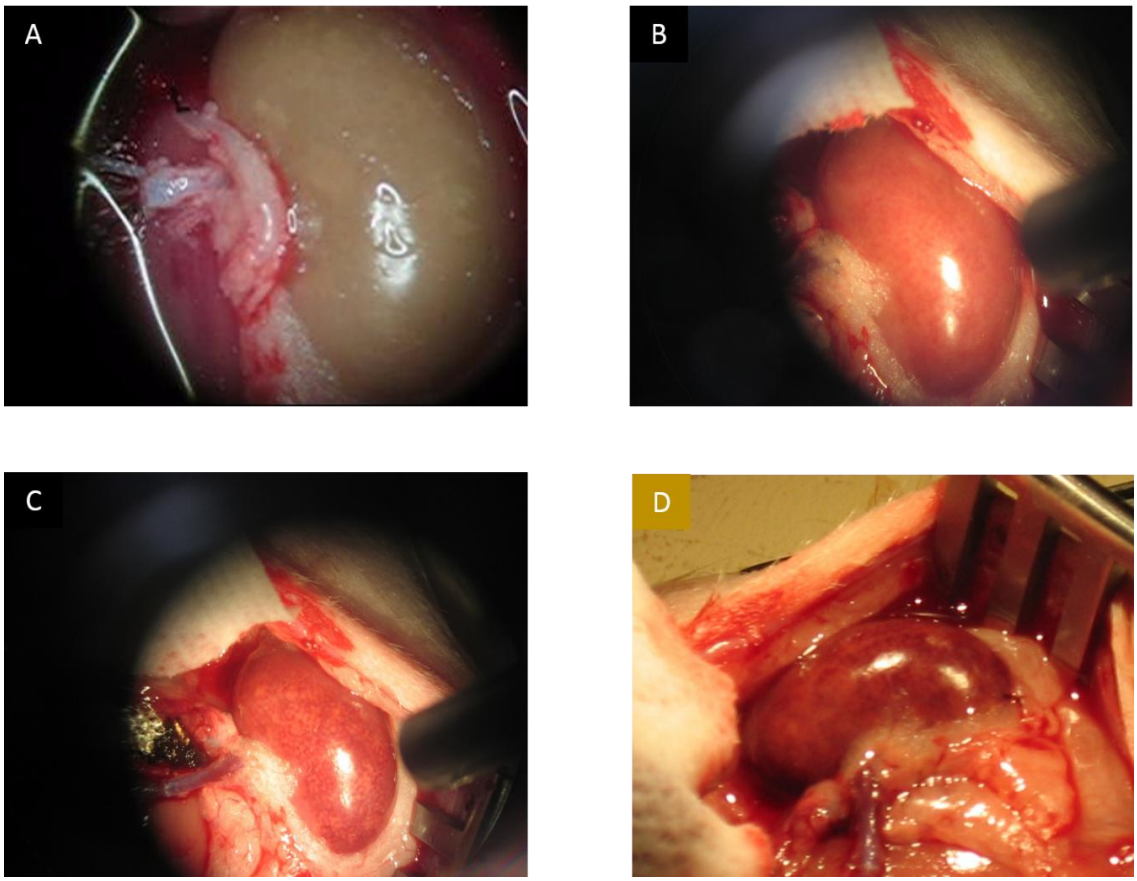


Figure 3.5: Photos of representative DA kidney transplanted into pre-sensitised Lewis recipient showing visible evidence of HAR. A: Isolated DA kidney, perfused with Soltran solution, prior to vascular anastomosis. B: DA donor kidney with healthy pink appearance two minutes after reperfusion begins. C: DA donor kidney at five minutes post-reperfusion showing a mottled appearance consistent with patchy reperfusion due to HAR. D: DA kidney allograft at ten minutes post-revascularization showing more extensive congestion associated with lack of perfusion.

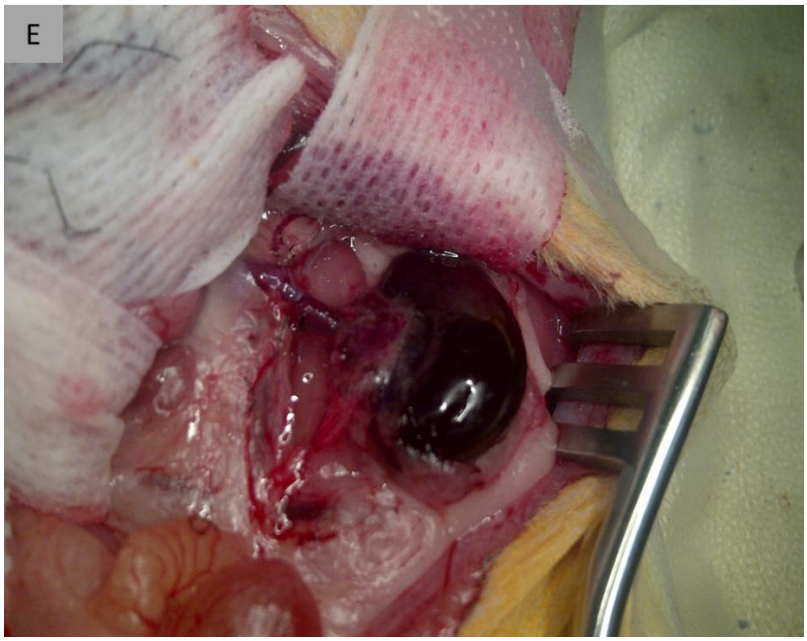


Figure 3.5. E: A DA kidney transplanted into a pre-sensitised Lewis recipient. In approximately 10% of the transplants, the donor kidney had turned black by the time the native nephrectomy had taken place (30 minutes after release of microsurgical clips), indicating thrombosis and infarction had occurred.

3.6 Histological Characteristics of Rejection in the Pre-sensitised Recipients

The cardinal features of HAR are based on the appearance of human transplanted kidneys. Typically these include the presence of neutrophils in the peritubular capillaries (PTC), microvascular thrombosis, haemorrhage and fibrinoid necrosis of the vessel wall. These features can be readily distinguished from those of T cell mediated rejection, which involve T cell and macrophage infiltration of the renal tubules and interstitial space. In order to verify HAR in this model the rejected kidneys in the hyperimmune group were examined histologically and compared with rejected grafts from the non-sensitised control group (Figure 3.6)

In the rejected kidneys from the pre-sensitised rats, typical features of HAR were present. These are illustrated in figure 3.6. In all, six kidneys in this group were examined, and representative results for three of these kidneys are shown. In contrast, the kidneys from the non-sensitised group (Figure 3.7) show extensive infiltration of the interstitial space and evidence of tubulitis consistent with T cell mediated rejection at around day 5 post transplantation. In addition, evidence of small vessel arteritis is present, as shown by arterial lymphocytic invasion and endothelial injury. Hence in the case of naïve recipients, the rejected kidney at day 5 post transplantation shows a mixed picture indicative of acute T cell mediate rejection and some features of early vascular (antibody-mediated) rejection, whereas the sensitised group of rejected kidneys show early and severe changes of HAR. The model developed for this study, therefore, appeared to be suitable to assess the impact of treatment for the prevention of HAR.

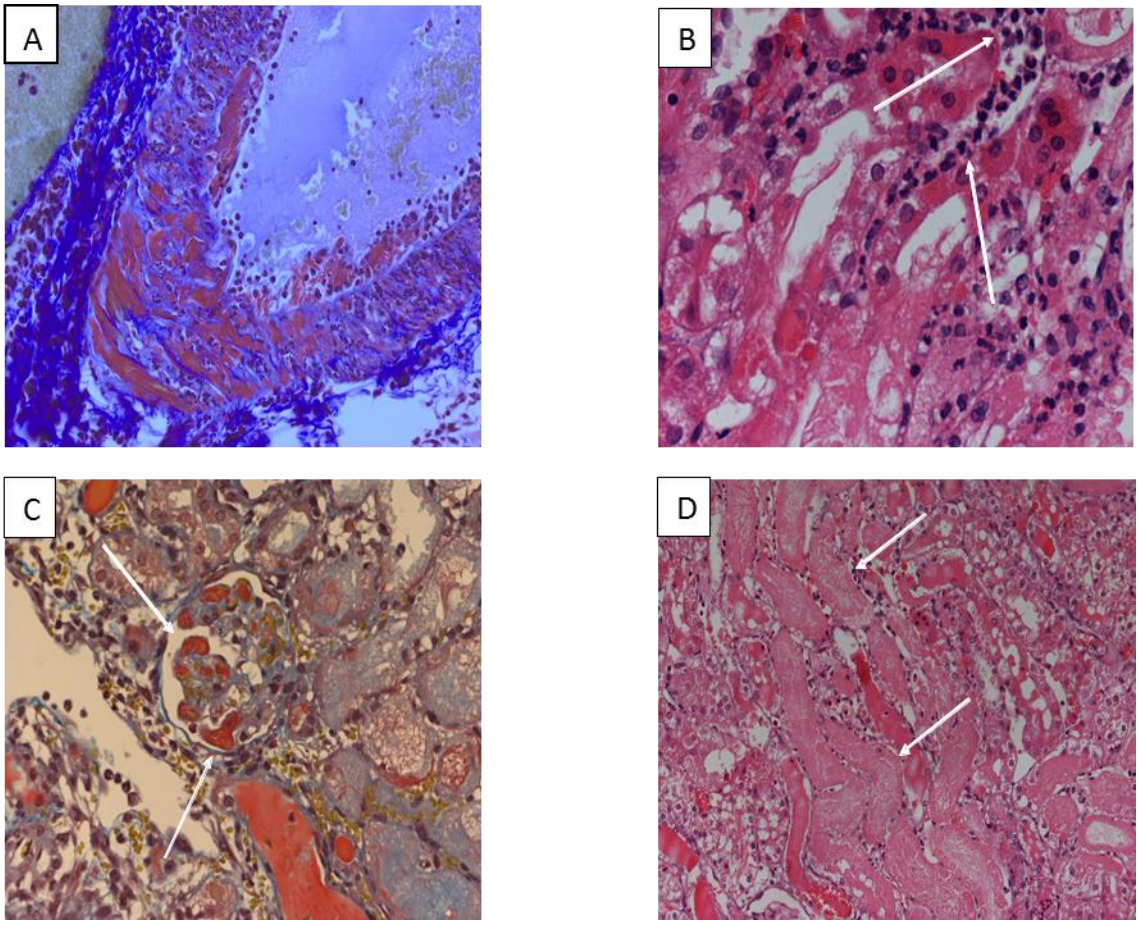


Figure 3.6: Morphological appearances typical of HAR represent the findings in micrographs from 6 kidneys. A: Histology of a representative DA kidney allograft taken from a pre-sensitised Lewis recipient at day 2 post-transplantation. Arterial fibrinoid necrosis of the vessel wall is seen (MSB; x200). B: Granulocytic cell infiltration of the PTC (H&E; x600). C: Multiple glomerular thrombi (MSB; x400). D: Appearances of a rejected kidney at day 1 post-transplantation, showing tubular necrosis and loss of tubule cell morphology (H&E; x200).

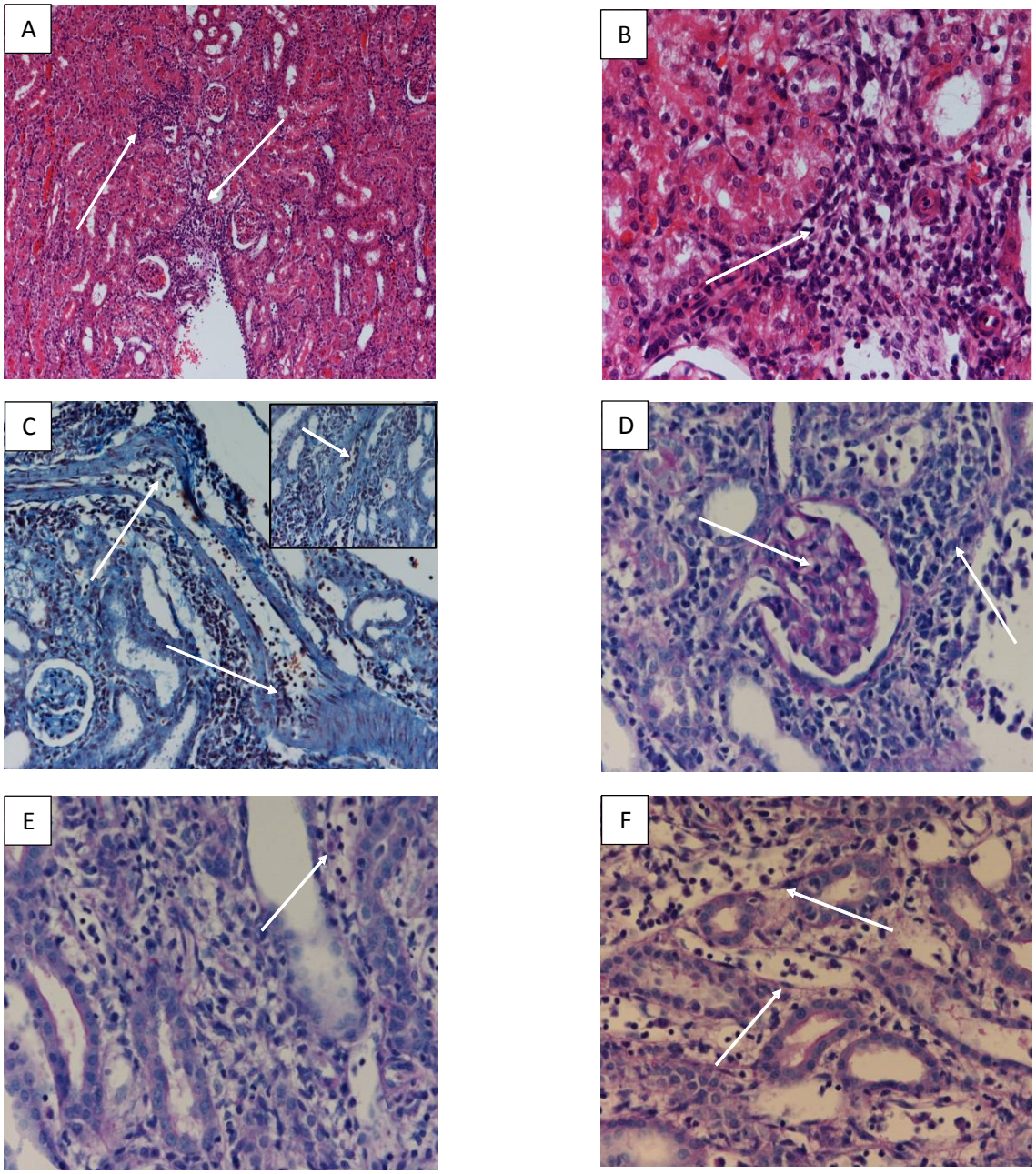


Figure 3.7: Histological findings in kidneys rejected by non-sensitised recipients (representative micrographs from a group of 6 kidneys). A: Histology of a representative DA kidney allograft from a non-sensitised Lewis recipient at day 5 post-transplantation showing extensive patchy lymphocytic infiltration (H&E; x100). B: Interstitial lymphocytic infiltration crossing the tubular basement membrane causing tubulitis (H&E; x400). C: Arterial wall infiltration by lymphocytes that are seen to adhere to the endothelium (MSB; x200). The inset shows endarteritis with penetration of lymphocytes in small artery (MSB; x400). D: Taken at day 7 post-transplantation,

histology showing both tubulitis and acute glomerulitis with mononuclear cells in the capillaries (PAS; x400). E, F: Numerous peritubular capillaries invaded with mononuclear leukocytes, revealing clear features of acute early AMR (PAS; x400).

3.7 Immunopathological Evidence for the Presence of Antibody in the Microcirculation of DA Kidneys transplanted into the Pre-sensitised Lewis Recipients

The identification of graft rejection due to the presence of DSA has become routine with the establishment of reliable detection of C4d, an inactive product of the complement classical pathway (Feucht HE et al., 1993 **57**). Its detection in transplant tissue correlates well with histological features of AMR in kidneys, such as the presence of neutrophils, monocytes and fibrinoid necrosis (Mauiyyedi S et al., 2002 **301**). C4d staining provides evidence of circulating complement fixing antibodies and it is considered a relatively specific and sensitive biomarker of AMR. The advantage of this small fragment generated from C4b is that, having an internal thioester moiety, it forms a strong covalent bond with nearby endothelial cells thus making it readily detectable.

To evaluate the presence of C4d in the rejected organs, immunofluorescent (IF) C4d staining on cryosections of transplanted kidney was performed. The frozen-IF technique was selected rather than paraffin-immunohistochemistry (IHC), since it is widely considered to be more sensitive (Seemayer GA et al., 2007 **302**). Diffuse C4d was detected in DA kidneys removed from the hyperimmune Lewis recipients at day 2 post-transplantation (Figure 3.8). Normal rat kidney showed no specific staining for C4d (Figure 3.9).

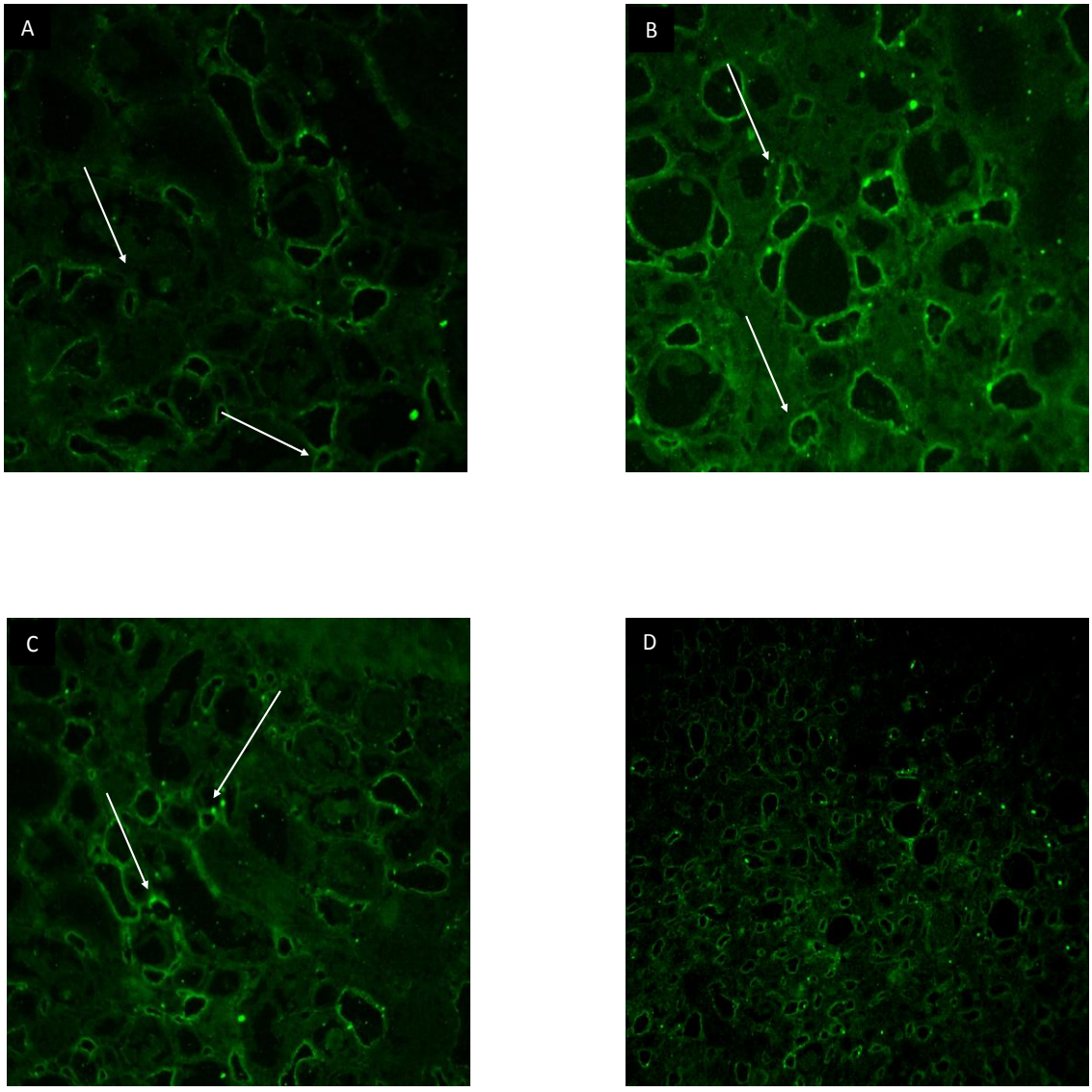


Figure 3.8. C4d immunofluorescent staining in a rejected DA kidney allograft from a pre-sensitised Lewis recipient. A, B: Linear deposition of C4d in dilated peritubular capillaries between ghost outlines of the renal tubules (x400). C: C4d staining in PTC (x200) D: Widespread staining of peritubular capillaries (x100).

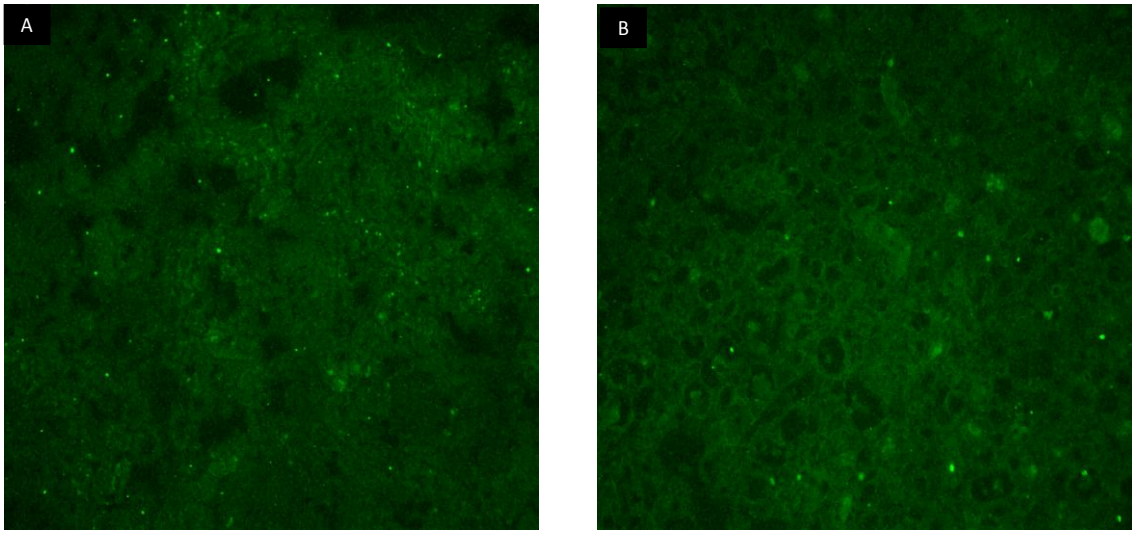


Figure 3.9. Negative controls for C4d immunofluorescent staining of DA kidneys. A: Frozen section of a normal on-transplanted DA kidney showing negative staining for C4d deposition (x100). B: DA kidney from transplanted sensitised Lewis recipient stained with isotype-matched antibody control (x100).

3.8 Discussion

The aim of the work described in this chapter was to establish an aggressive model of AMR in rat kidney transplants, comparable to that seen in human kidney transplants in the presence of DSA. Due to the use of cross-matching techniques this type of rejection is very rare in the clinic. The rationale for the development of this model and associated research was to provide a severe challenge for the development of a therapeutic strategy that ultimately could be applied in highly sensitised patients who are presently considered non-transplantable. In order to confirm the suitability of this model for such purposes, the following characteristics (of HAR) would be required:

1. Donor-specific IgG antibody in the serum
2. Immediate onset of rejection
3. Histological evidence of the typical lesion
4. C4d deposition on the peritubular capillaries

A prerequisite for a successful model was to use an appropriate donor/recipient rat strain combination, so that upon sensitisation the recipient would produce high titre donor-specific antibodies. The method of sensitisation of the recipients was based on a study in which three successive allogeneic skin grafts led to HAR in a rat model of cardiac transplantation (Brauer RB et al., 1995 **275**). It was concluded form the cardiac study that multiple skin immunizations were necessary for the production of DSA whose level and potency were so great that they could elicit a different type of rejection, as compared with a naïve recipient. This method was pioneered by Guttmann during studies involving a series of heterotopic rat cardiac allografts in 25 different strain combinations (Guttmann RD et al., 1974 **303**). These experiments showed that HAR after recipient sensitisation only occurred in specific donor/recipient rat-strain combinations. Interestingly, organs from donors with the $RT1^a$ haplotype, expressed by the ACI rats, consistently induced this phenotype. Thus, it was concluded that to reproduce the transplant setting in which the donor organ is highly immunogenic, the rat donor/recipient combinations have to be carefully selected. Furthermore, these studies are important because they showed the validity of using rats in transplantation models. In this particular case, the heart transplantation between ACI and PVG rats (also used by Brauer RB et al., 1995) proved to be a well suited model for such clinical type studies. For the purpose of my study, the well-established, high responder DA to Lewis

strain combination was used. DA rats share the same highly immunogenic, $RT1^a$ haplotype, and represented an appropriate donor strain since ACI rats are not available in the UK.

Having decided on the appropriate rat strain combination, recipient Lewis rats were pre-sensitised with three DA full-thickness skin segments. Prior to kidney transplantation, all three skin grafts had been completely destroyed and were replaced by scar tissue upon visual inspection. This sensitisation protocol correlated with flow cytometric evidence that the sera from Lewis rats contained high titre DA specific IgG antibody. Although IgM fixes complement and has been associated with HAR in this model it was not systematically measured during the immunization schedule I used. IgM is relatively short lived, and considering the long duration of the sensitisation protocol, it is likely all IgM production would have switched to IgG, by the time the kidney transplantation was carried out. In addition, the observation that IgG antibodies are associated with poor prognosis and severe rejection, serum IgM analysis (McKenna RM et al., 2000 **307**) was not included in this model.

The titre and expected affinity of the alloreactive antibodies generated by the pre-sensitisation procedure was such that it was able to significantly accelerate rejection resulting in an MST of 2 days compared with 5 days for non sensitised recipients. By definition, HAR occurs within minutes to hours post revascularization of the organ. In this model, rejection (death) on day 1 or 2 post reperfusion is misleading. Whereas in heterotopic cardiac models, loss of graft function is immediately detectable by loss of heart-beat upon palpation, the cessation of kidney allograft function requires at least 24 hours before the animal succumbs. However, as shown by direct inspection of the transplanted organ at the time of surgery, the loss of blood flow and onset of tissue infarction was instantaneous, occurring within minutes of the microvascular clips being released. These kidneys had ceased function long before the death of the recipients, which required some time before they succumbed to renal failure. Thus, the immediate circulatory collapse and characteristic changes in tissue morphology in the presence of a high titre of donor-reactive IgG antibody can be reasonably concluded to be due to HAR.

Positive C4d staining in the PTC is now an accepted diagnostic criterion in the recognition of AMR and has consistently been associated with poor allograft outcome.

C4d is formed following the cleavage of complement factor C4 into C4b and thereafter into an inactive form and degraded to C4d, attached by a covalent bond on the activation site. As a result, it remains bound for a prolonged period of times and can be easily detected with IF and IHC protocols, serving as a marker of AMR. C4d has proven to be a marker of active AAMR and follow up biopsies in a patient survey revealed that C4d could be detected up to 58 days post transplantation following successful treatment of ongoing AMR (Koller H et al., 2004 **305**). Detection of C4d has, in part, also solved the problem of immunohistological detection of IgG. IgG deposition on tissue has not proved a reliable marker of AMR in many reports, and this may be related to rapid shedding from the graft endothelial cells due to its weak interaction being mediated by weak van der Waals bonds (Cohen D et al., 2012 **60**) or possibly internalization of bound antibody by the endothelial cells. Furthermore, the detection of C4d, most importantly, shows involvement of the complement system, and although C3d is also deposited at sites of antibody binding, its presence is non-specific (Colvin RB, 2007 **12**) as C3 breakdown is central to all complement activation pathways. C3 is often detected on the tubular basement membrane, which is not a target for AMR. For this reason, in this study, confirmation of DSA and the involvement of complement relied upon C4d staining.

Snap-frozen tissue was stained using standard immunofluorescent techniques (IF) and was found to be positive for C4d on the PTC of the kidneys transplanted into the pre-sensitised Lewis rats. However, it is important to note that at the time of this investigation, no anti-rat C4d antibody reagent was commercially available. Newly generated anti-rat C4d antibody (courtesy of Dr Sarah de Freitas) was tested in this model. This antibody was generated in-house specifically against the peptide sequence for rat C4d. As previously stated, positive staining was observed and this pathology was confirmed by Dr Catherine Horsfield. Nonetheless, cautious interpretation of these data is required since subsequent staining with different batches of this new antibody, did not reproduce the result (confirmed by colleagues). Currently, new reagents have become available (Hycult biotech) and could be used in future studies.

In conclusion the data reported in this chapter meet the criteria proposed at the outset, and appear to present a formidable challenge required for this study and described in more detail in the following chapters. The issue to be explored in these chapters is

whether intervention with complement or coagulation regulators can modify the pathological process and clinical course associated with high titre DSA at the time of transplantation.

Chapter 4 – Impact of Therapeutic Complement Regulator APT070 (Mirococept) on HAR

4.1 Introduction

Chapter 3 described the immunological and pathological features of an aggressive model of hyperacute antibody-mediated rejection akin to HAR in human kidney transplantation. The present chapter explores the hypothesis that by increasing the local concentration of complement regulator on the endothelial cell surface by therapeutic means, the endothelial damage mediated by complement will be reduced and graft survival will be enhanced. Such a cell-protective (cytoprotective) approach is made feasible by the advent of 'cytotopic' technology, described by Dr Richard Smith and colleagues (Smith RAG, 2002 **286**). This method for localizing therapeutic agents on the cell surface was outlined in the Chapter 1. The prototype, Mirococept (APT070), is a derivative of soluble recombinant human complement receptor 1 (sCR1) attached to a membrane binding tail. The latter is composed of two parts: a membrane inserting myristoyl which forms hydrophobic interactions with the phospholipid membrane; and a positively charged peptide sequence which allows electrostatic interactions to occur with phospholipid heads which are negatively charged. The generation of membrane-binding APT070 through the process of the 'myristoyl-electrostatic switch paradigm' (Smith RAG, 2002 **286**) makes cytotopic therapy achievable, especially in the transplant setting, where donor organs can be treated prior to transplantation (Pratt et al., 2003 **290** Patel et al., **291**). It offers a strategy that is not only highly specific to the organ where inflammation is taking place, but also avoids interference with systemic complement (Mollnes TE et al., 2006 **287**). Membrane insertion increases retention time (reduces clearance) and the fact that APT070 is a small molecule (24kDa) in comparison to native CR1 (250kDa) (Dodd I et al., 1995 **285**), makes it less immunogenic in rodents (Patel H et al., 2006 **290**). APT070 has been used in a variety of animal models such as rat models of acute vascular shock (Smith RAG, 2002 **254**), experimental rheumatoid arthritis (Linton SM et al., 2000 **256**), myocardial infarction in pigs (Banz Y et al., 2007 **257**) and ischaemia-reperfusion injury in rat kidney transplants (Pratt et al., 2003 **290**; Patel et al., 2006 **291**). These studies showed that APT070 is well tolerated and has impressive cytoprotective effects. Furthermore, a Phase 2a kidney transplant study

showed that the technique can be transferred to man (Smith R/Sacks S unpublished data)**.**

The rationale for using complement-based approaches to prevent or suppress HAR is supported by therapeutic animal and clinical studies. Previous studies in mice indicate that inhibition of the terminal complement components (C5b-9 and C5a) using with an anti-C5 monoclonal antibody can ameliorate HAR (Wang H et al., 2007 **276**). Wang et al. showed that anti-C5 in combination with immunosuppressive agents could prolong the survival of cardiac allografts transplanted into hyperimmune mouse recipients. Similarly, kidneys transplanted into pre-sensitised recipients injected with C5mAb achieved prolongation of graft survival compared to untreated control mice (Rother R P et al., 2008 **277**). In addition, promising results were obtained in a small number of sensitised human recipients who received anti-C5 antibody (Eculizumab) post-transplantation. In a proportion of these patients, the incidence of AMR was decreased (Lonze BE et al., 2010 **263**; Stegall MD et al., 2012 **264)**. The aim of the investigations described in this chapter was to determine whether APT070 can protect treated rat kidney against HAR. This is the first study using such a stringent model of AMR in allotransplantation in which complement is targeted locally rather than systemically.

4.2 Experimental design

Before proceeding with the *in vivo* evaluation of the effects of Mirococept in the rat hyperimmune transplant model, verification experiments were carried out for characterization of the reagents. Functional assays were performed to confirm the capacity of APT070 to inhibit complement activation *in vitro* and bind to cell surfaces, *in vivo*. The reagents utilized for this purpose were the tailed SCR1-3 (APT070) and untagged SCR1-3 (APT154) as control, (kindly provided by Dr R Smith, KCL).

Inhibitory potency of APT070 was confirmed *in vitro* using a standard haemolytic assay. In this assay, antibody sensitised sheep erythrocytes activate complement and cause cell lysis. The ability of APT070 to inhibit complement activation and reduce cell lysis provides a measure of therapeutic functionality. The contribution of membrane binding to APT070-mediated protection was determined by comparison of the functionality using the untagged soluble peptide, APT154. *In vivo* experiments were subsequently carried out to confirm that DA kidneys treated with APT070 could bind

this inhibitor, with an appropriate tissue distribution following intra-arterial administration, as previously demonstrated (Patel H et al., 2006 **291**). Finally, after clarifying the inhibitory and binding efficacy of Mirococept, APT070-treated organs were transplanted into hyperimmune Lewis recipients, to determine the impact on graft survival.

The *in vitro* functional analysis of Mirococept was carried out by performing a well-established, classical pathway haemolytic assay (Morgan BP, 2000 **306**). Rat serum was used as the source of complement which was incubated with antibody sensitised sheep red blood cells. This interaction activates complement by the classical pathway through the formation of antigen-antibody complexes and results in haemolysis due to the formation of the MAC (C5b-9) in the red blood cell membrane. To determine whether the addition of APT070 protects the sheep red blood cells from lysis, haemolytic inhibition (% HI) was determined. This was compared to the non-tagged APT154.

To establish the distribution of APT070 in the donor kidney following intragraft delivery, DA kidneys were perfused with a solution containing APT070 and the treated organs were transplanted into *syngeneic* recipients. Immunohistochemistry was performed on tissue sections of frozen kidney removed at 0.5 and 24 hours post-transplantation. Control kidneys treated with the non-tagged agent APT154, were removed at the same time points and used for the analysis.

Finally, after completion of % HI *in vitro* experiments and binding experiments *in vivo*, both inhibitory and control reagents were used in the hyperimmune model of allotransplantation. DA kidneys were perfused via the renal artery with Mirococept at two different doses, 40 or 80 μg/ml, before being transplanted into hyperimmune Lewis recipients. This dose regimen was based on an earlier dose schedule that was successful in reducing ischaemia/reperfusion injury (Patel H et al., 2006 **291**). The organ perfusion and renal transplantation procedure was adapted from that described for a rat model of chronic kidney transplant rejection between Fischer F344 ($RT1^{Iv1}$) and Lewis ($RT1^{I}$) rats (Pratt J et al., 2003 **290**). The control group was perfused with non-tailed CR1, APT154. Transplant survival in recipient rats was measured in conjunction with evaluation of renal function by daily analysis of serum blood urea nitrogen (BUN). Graft pathology was examined to determine any differences between the inhibitor- and control-treated groups with respect to the development of HAR. Graft rejection was

taken as the time of death of the recipient (or time of sacrifice as determined by Home Office regulations) which was always accompanied by high serum BUN (i.e. >35 mmol/L).

4.3 *In vitro* activity of APT070 as a Complement Inhibitor

The functional activity of APT070 as a complement inhibitor was verified *in vitro* and compared against the untagged control reagent APT154, using a standard haemolytic assay. In this assay, antibody-sensitised sheep red blood cells were incubated with serum from a normal DA rat at a pre-defined dilution, to which had been added APT070 to give different final dilutions. APT070 was found to inhibit complement-mediated cell lysis in a dose-dependent manner. The concentration of APT070 found to inhibit classical pathway-induced haemolysis by 50% (IH50) was ~1.5 nM. The protective effect of the untagged control agent APT154 was substantially less, since the IH50 for APT154 was found to be ~ 85 nM (Figure 4.1). In other words, the potency of the tailed regulator in functional inhibition of complement was about 57x that of the non-tailed regulator

In summary, this experiment confirmed APT070 to be a potent inhibitor of the classical pathway of complement activation in rat serum. The equivalent therapeutic non-tailed therapeutic fragment was substantially weaker in its ability to inhibit complement activity on the sensitised sheep red cell surface. These data demonstrate a distinct advantage of the cytotopic technology used to attach the complement regulatory fragment of APT070 to the target-cell surface.

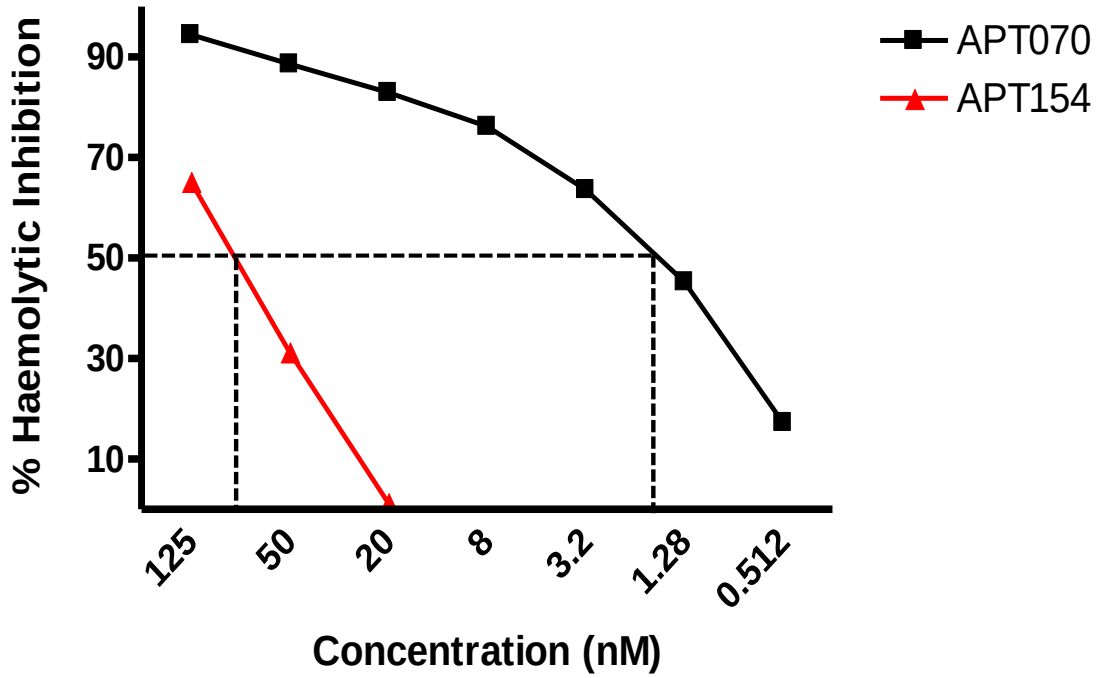


Figure 4.1 **%** Haemolytic Inhibition shows APT070 inhibition of complement-mediated cell lysis in a dose-dependent manner and IH50 ~1.5 nM in rat serum. The untagged control reagent APT154 shows less inhibitory effect, with IH50 ~85 nM in rat serum (p=0.0021). A titration of each reagent in rat serum was incubated with antibody-sensitised sheep red blood in a haemolytic assay. % Haemolytic inhibition was calculated.

4.4 Localisation of APT070 within treated Rat Kidney Isograft

A key objective of my study was to ensure the delivery of the tagged complement regulator APT070 to the renal vascular endothelium after intra-arterial injection of carrier solution containing the APT070. Retention of APT070 at this site was considered essential to the proposed therapeutic action of APT070 in reducing the damage caused by HAR. In order to investigate the distribution of APT070, perfusion of DA kidneys with Soltran containing either 40 μg/ml of APT070 or untagged APT154 was carried out. Treated organs were transplanted into syngeneic (DA) recipients, in order to first examine the distribution of the therapeutic agent in the absence of an alloimmune response. Analysis involved immunohistochemistry on frozen tissue sections prepared from the transplanted kidney after it had been removed from the recipient. The time points analyzed were 0.5 and 24 hours post transplantation, so as to cover the time span of rejection identified in the study of allogeneic hyperimmune transplants (Chapter 3). As shown in figure 4.2 binding of APT070 following perfusion was uniformly detected in a glomerular capillary pattern indicating that APT070 was attached to the endothelial surface 0.5 hours post transplantation. Less intense staining was detected on some peritubular capillaries. Staining tissue perfused with control reagent APT154 was negative at the same time point.

In contrast to the bright staining in the glomeruli of tissue harvested after 30 minutes, at 24 hours post transplantation, the complement inhibitor APT070 was no longer detectable on the endothelium. However, it was present, at low intensity in focal regions of the kidney. More specifically, positive staining seemed to localise on the apical side of the renal tubules rather than the tubular epithelium itself. Negative staining was observed for the tissue perfused with untagged APT154 (Figure 4.3).

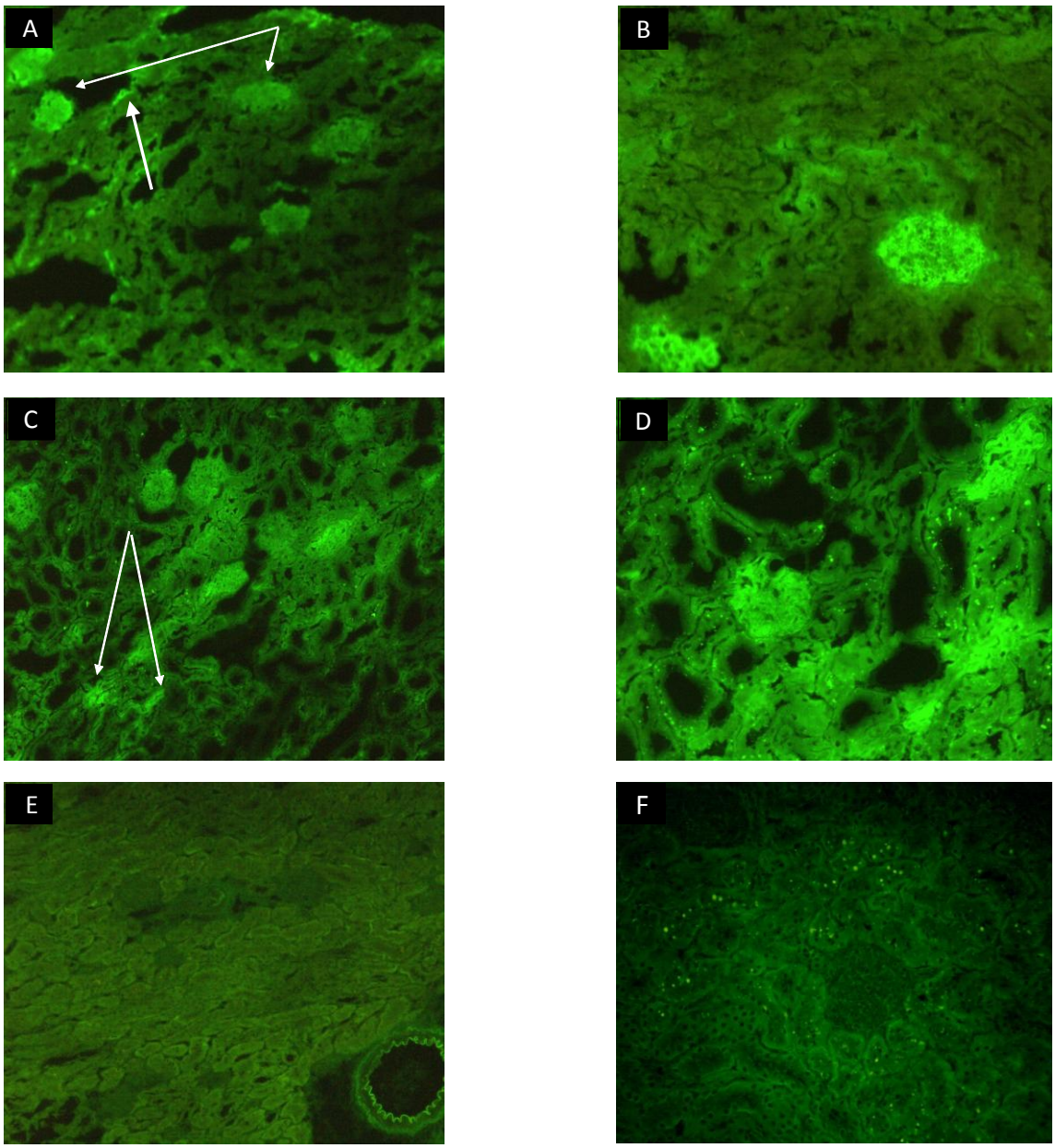


Figure 4.2: Immunohistochemical staining with antibody against human CR1 following perfusion of DA donor grafts with APT070 (n=2) or control untagged reagent APT154 (n=2) prior to transplantation into syngeneic recipients. Tissues were analysed at 0.5 hours post transplantation. A, B: Heavy staining within the glomeruli is in the pattern of the glomerular capillary tuft. C, D: Positive deposition of APT070 is detected on both the glomerular and peritubular capillary walls consistent with endothelial staining shortly after perfusion. E, F: Negative staining of DA donor kidney perfused with non-tailed agent APT154. Representative images were taken at x100 (A, C, E) and x200 (B, D, F).

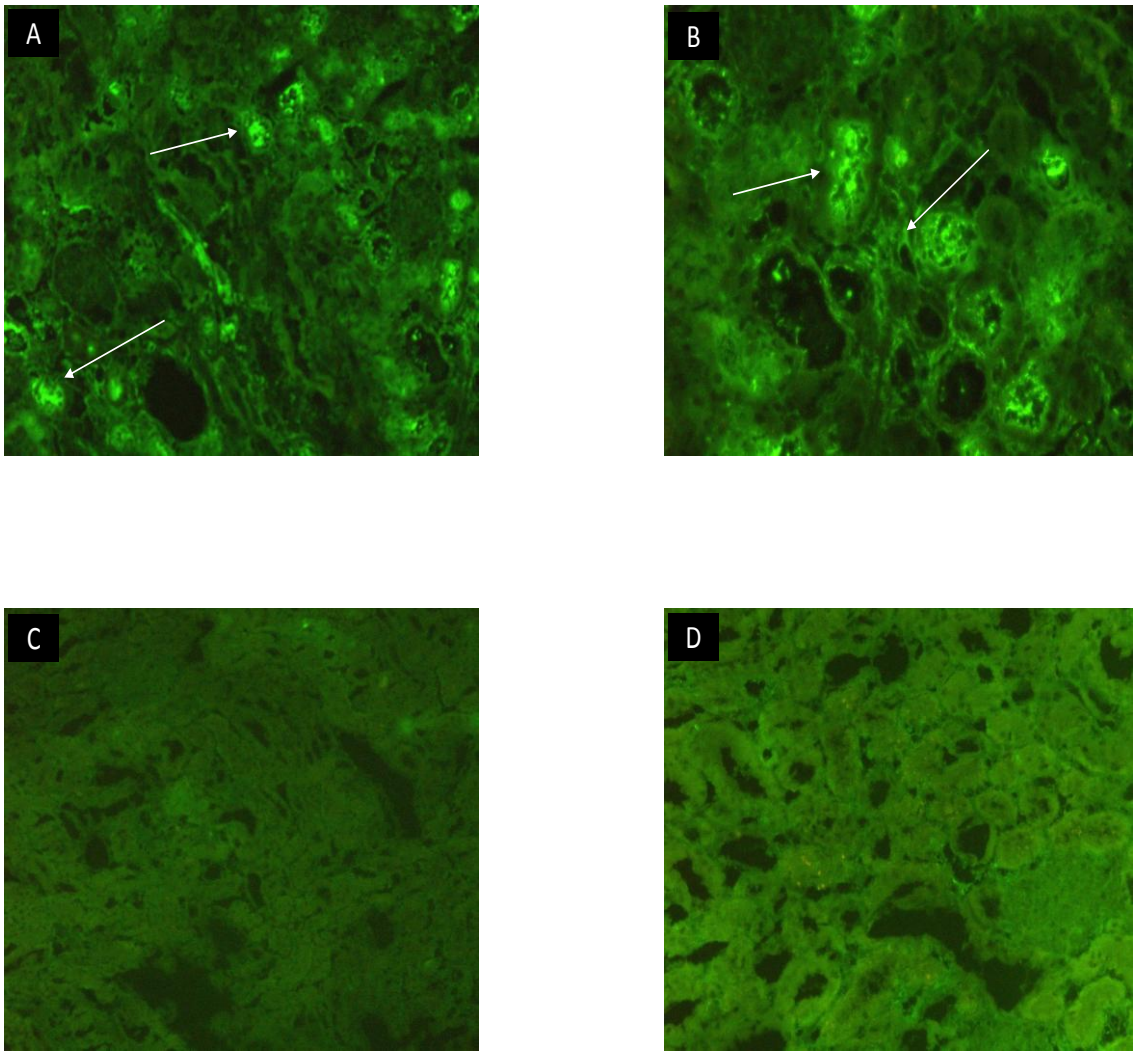


Figure 4.3: Immunohistochemical staining for human CR1 in DA donor grafts treated with APT070 (n=2) or control reagent APT154 (n=2) prior to transplantation into syngeneic recipients. Tissues were analysed at 24 hours post transplantation. A, B: Focal distribution of APT070 is observed on the renal tubular epithelium and more specifically in the lumen of the proximal and distal tubules. C, D: Negative staining of DA donor kidney perfused with non-tailed APT154. Representative images were taken at x100 (A, C) and x200 (B, D).

4.5 Graft Survival in Pre-sensitised Lewis Recipients following Treatment of the DA donor Kidney with APT070

Using *in situ* perfusion of DA donor kidneys with APT070 or control reagent APT154, the effect of the complement inhibitor on graft survival was monitored and requirement for membrane binding was determined. Kidneys pre-treated with 40 μg/ml of APT070 did not exhibit prolongation of survival compared to the untagged SCR1-3 (APT154). Both groups showed rapid rejection, of similar speed and severity as had been observed in preliminary experiments using Soltran perfusion fluid alone to treat the donor organ transplanted into hyperimmune recipients (chapter 3). In the APT070-treated group (MST 1.4 days) 60% of animals survived past 24 hours (3 of 5) but none survived beyond the 48 hour time point. A similar trend was observed for the recipients of grafts treated with control reagent APT154 (MST 1.2 days). 75% of the animals survived past 24 hours post-transplantation (3 of 4) whereas, again, none of the animals lived past 48 hours (Figure 4.4A). Graft loss was defined as the death of the recipient or its requirement to be culled due to obvious distress, correlating with high serum BUN.

Following these results with 40 μg/ml of the reagent, the dose of APT070 was increased to 80 μg/ml in a further experiment using an identical transplant procedure. Despite doubling the dose of APT070, there was no clear impact on graft survival, when compared to APT154 control-treated kidneys in the same experiment, or to Soltran-treated kidneys in the earlier baseline study (chapter 3). 55.5% of animals that received APT070-treated grafts (MST 1.4 days) (5 of 9) survived to 48 hours post-transplantation compared with 50% of Lewis rats that received APT154-treated kidneys (MST 1.5 days post-transplantation) (2 of 4) (Figure 4.4B). Thus, treatment even with a higher dose of the complement inhibitor did not extend survival beyond 48 hours post transplantation.

These data indicated that in this very aggressive model of hyperimmune kidney rejection, inhibition of complement with the membrane-localizing inhibitor, Mirococept, perfused at two different doses, 40 and 80 μg/ml, provided no benefit in survival compared to its control reagent, APT154.

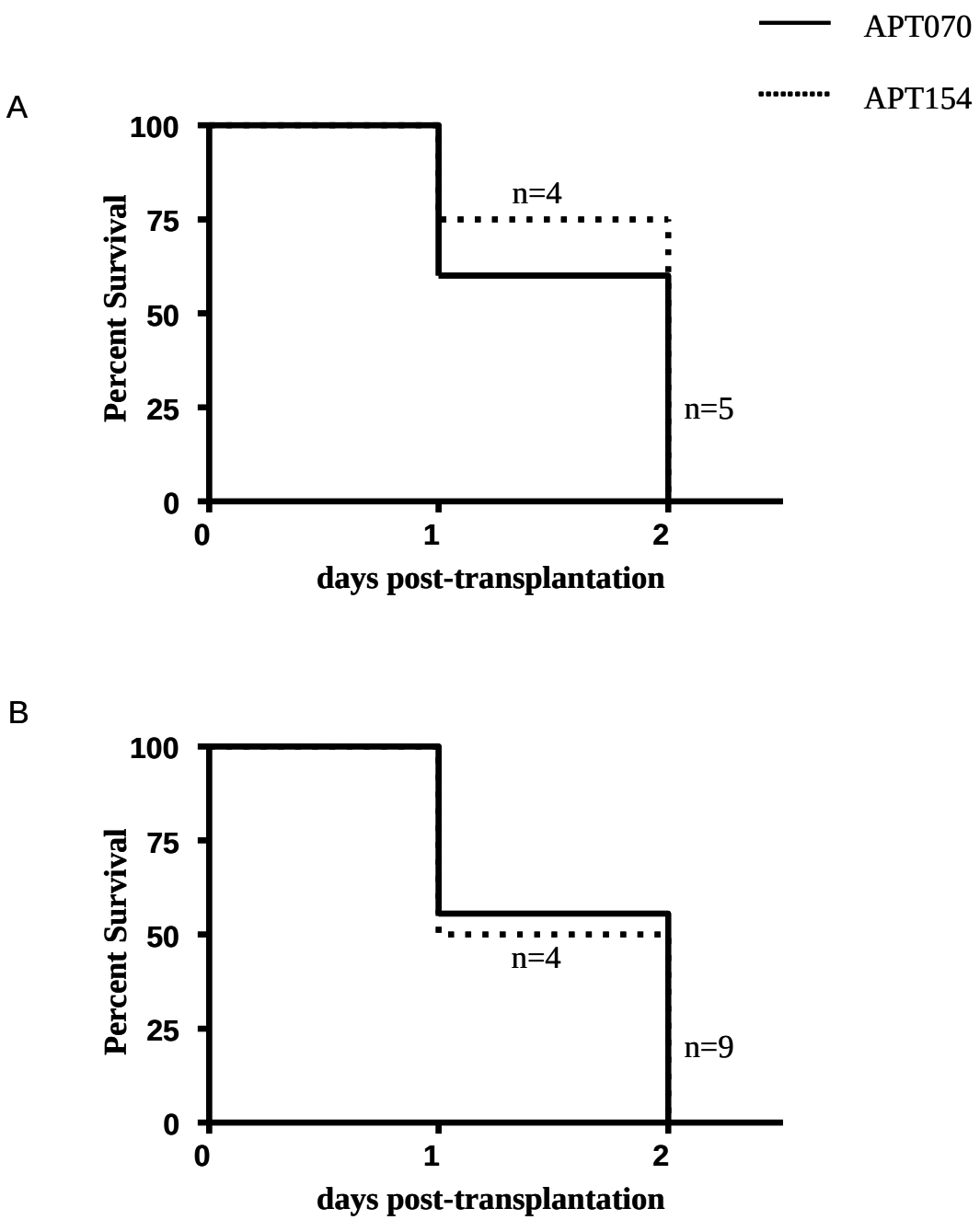


Figure 4.4: Graft survival following transplantation of DA kidneys treated with APT070 or control agent APT154 into pre-sensitised Lewis recipients. A: APT070 treatment of donor organs with a dose of 40 μg/ml transplanted into pre-sensitised rats (n=5) achieved no significant prolongation of survival compared to the untagged APT154 control group (n=4) (p=0.6547). B: APT070 treatment of donor organs with a dose of 80 μg/ml transplanted into pre-sensitised rats (n=9) achieved no significant prolongation of survival compared to the APT154 control group (n=4) (p=0.8586).

4.6 Renal Function of Pre-sensitised Lewis Recipients after transplantation with APT070-treated DA Kidney

In the same groups of experimental rats described in Section 4.5 (with 40 or 80 ug/ml of therapeutic agent or control applied to the transplant), renal function in the Lewis recipients was monitored daily following transplantation until the time of the graft rejection. The results are shown in figure 4.5.

At 24 hours post-transplantation, the BUN levels for pre-sensitised recipients of DA kidneys pre-treated with 40 μg/ml of APT070 were very similar to those of the APT154 control-treated group, (i.e. >35 mmol/L). At 48 hours post-transplantation, the BUN levels for the surviving rats in both APT070 and APT154-treated groups remained high (> 35 mmol/L). Therefore no significant benefit was observed (Figure 4.5 A, B), in terms of reducing BUN towards levels in healthy rats (approximately 10 mmol/L, data not shown).

In the group of Lewis recipients where the DA donor organ was treated with 80 μg/ml of APT070 or APT154, BUN levels showed a trend toward improved renal function in the APT070 group compared to the APT154 group, at both 24 and 48 hours post-transplantation (Figure 4.5 C, D). However, these results were not statistically significant.

These data showed that pre-treatment of DA donor kidney with either 40 or 80 μg/ml APT070 before transplantation into hyperimmune Lewis recipients had no major impact on renal function at both 24 and 48 hours post-transplantation compared to treatment of the organ with the untagged reagent.

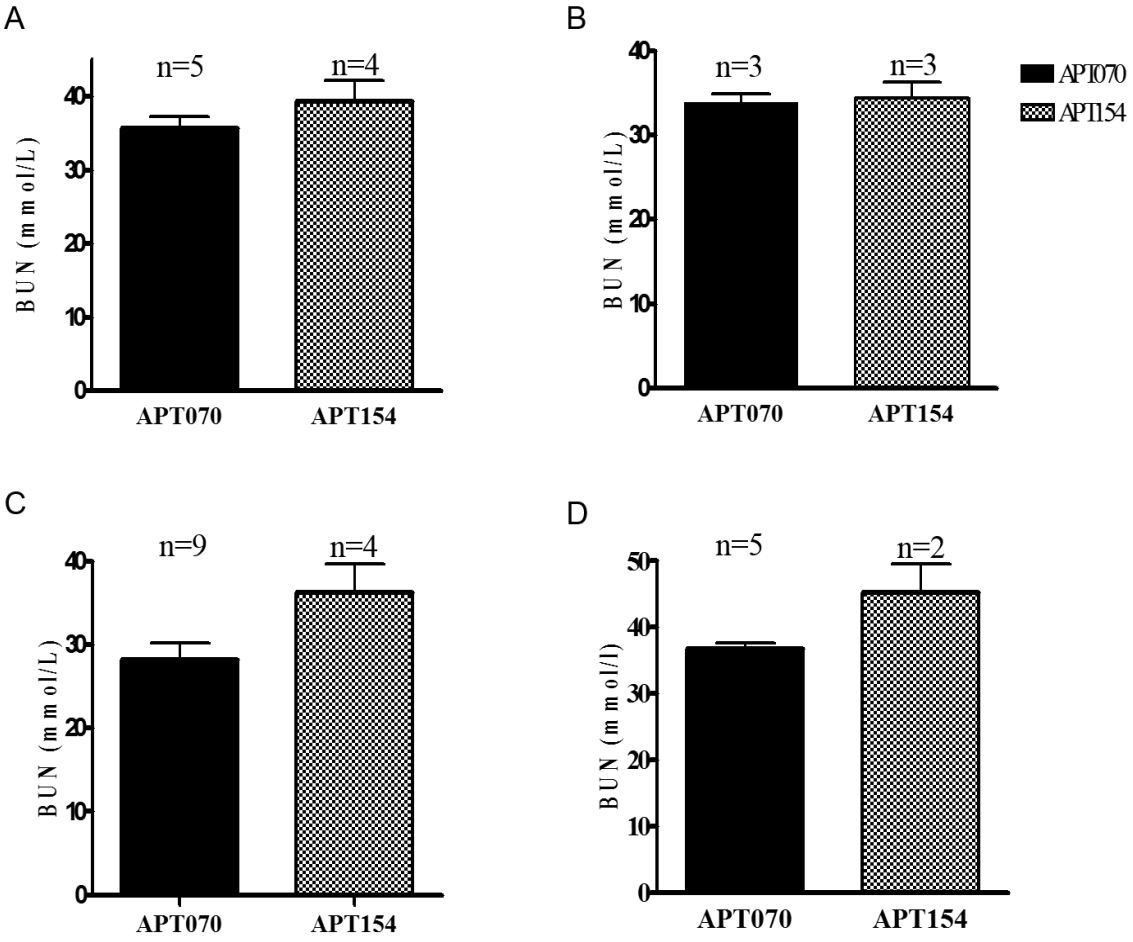


Figure 4.5: BUN measurements as an indicator of renal transplant function. A, B: APT070 or APT154 40 μg/ml used to treat the donor organ: A: Function at 24 hours post-transplantation (p=0.7302). B: Function at 48 hours post-transplantation (p=0.400). C, D: APT070 or APT154 80 μg/ml used to treat the donor organ: C: Function at 24 hours post-transplantation (p=0.110). D: Function at 48 hours post-transplantation (no statistical analysis was possible due to limited recipient numbers).

4.7 Renal Transplant Pathology of the Treatment and Control Groups

Despite no clear prolongation of survival and only moderate improvement in renal function in Lewis recipients of APT070-treated DA kidneys, graft pathology was assessed. The purpose was to determine whether any improvement in the histological injury associated with APT070 treatment could be identified by histological analysis. As illustrated in figure 4.6, no morphological differences were observed between APT070 and APT154-treated kidneys when the dose of reagent was 40 μg/ml. The treated DA kidneys exhibited extensive tissue necrosis and widespread thrombosis. Massive thrombosis in glomerular and arterial endothelium was evident (Figure 4.6).

At the higher dose of APT070, DA donor kidneys transplanted into pre-sensitised Lewis recipients revealed subtle differences when compared to APT154 control-treated organs. APT070-treated organs, although infarcted, had dispersed patches of preserved tissue, which was not detected in the APT154 group. Furthermore, small vessel thrombosis was prominent in the APT070-treated group, whereas in the control group, the coagulopathy extended to the larger vessels, which showed fibrinoid necrosis of the vessel wall (Figure 4.7).

Figure 4.8 shows that these intense thrombotic phenomena were accompanied by extensive inflammatory infiltration around the peritubular capillaries of both APT070- and APT154-treated grafts at day 1 post-transplantation.

Nonetheless, the kidney injury observed in both treatment groups was extensive. Therefore, treatment with APT070 alone, in the stated doses, was insufficient to prevent HAR in this highly aggressive model.

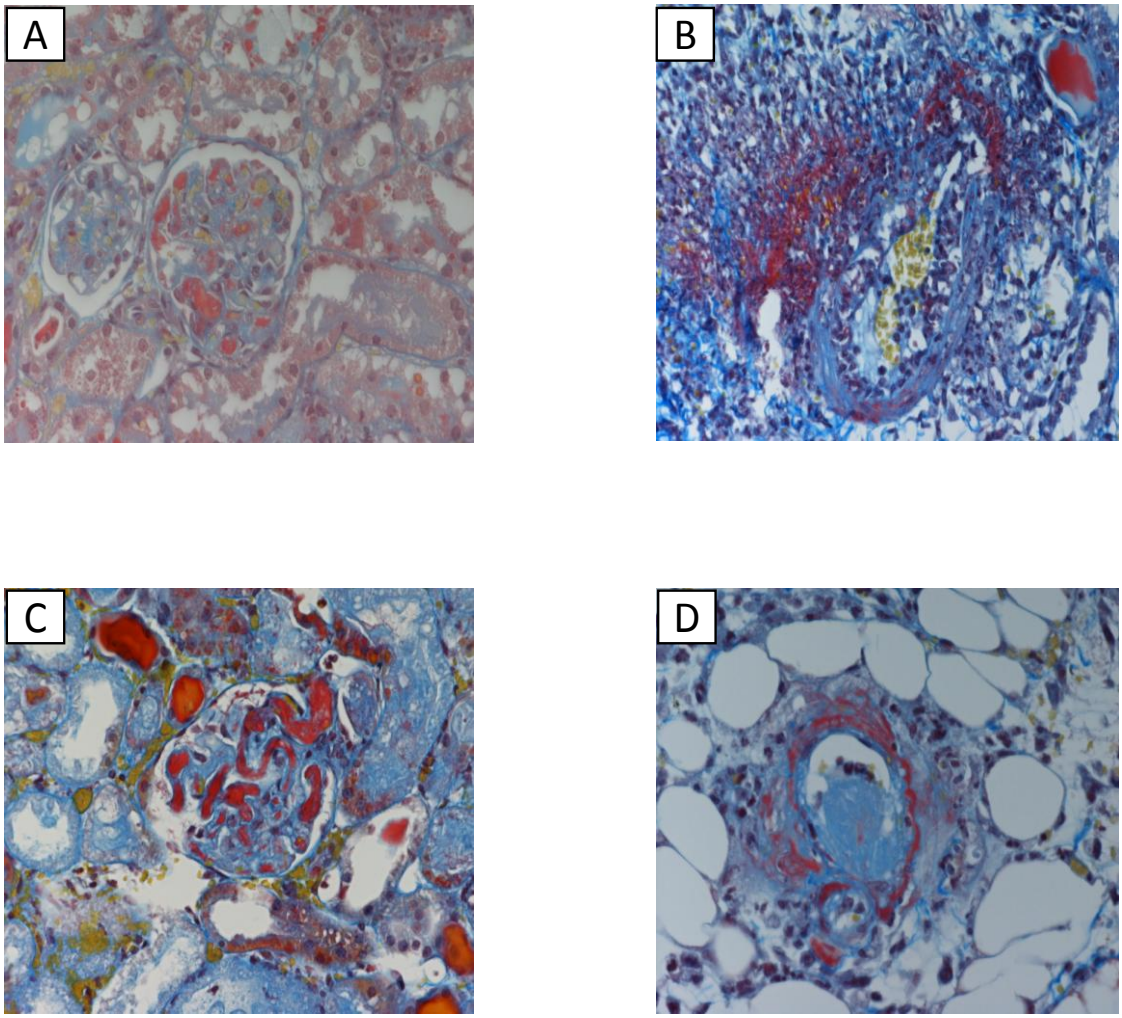


Figure 4.6: MSB-stained histological sections of rejected transplants at x400 magnification. Donor kidneys treated with 40 μg/ml of APT070 or control APT154. A: APT070-treated graft removed from a pre-sensitised Lewis recipient at day 1 post-transplantation showing glomerular thrombosis. B: Artery of an APT070-treated graft showing fibrinoid necrosis at day 2 post-transplantation. C: APT154-treated graft from pre-sensitised Lewis recipient at day 1 post-transplantation showing presence of glomerular endothelial thrombi. D: Fibrinoid necrosis of the vessels in an APT154-treated graft at day 2 post-transplantation. The pictures are representative of five APT070-treated kidneys and four APT154-treated kidneys examined after removal from the recipient at the times indicated.

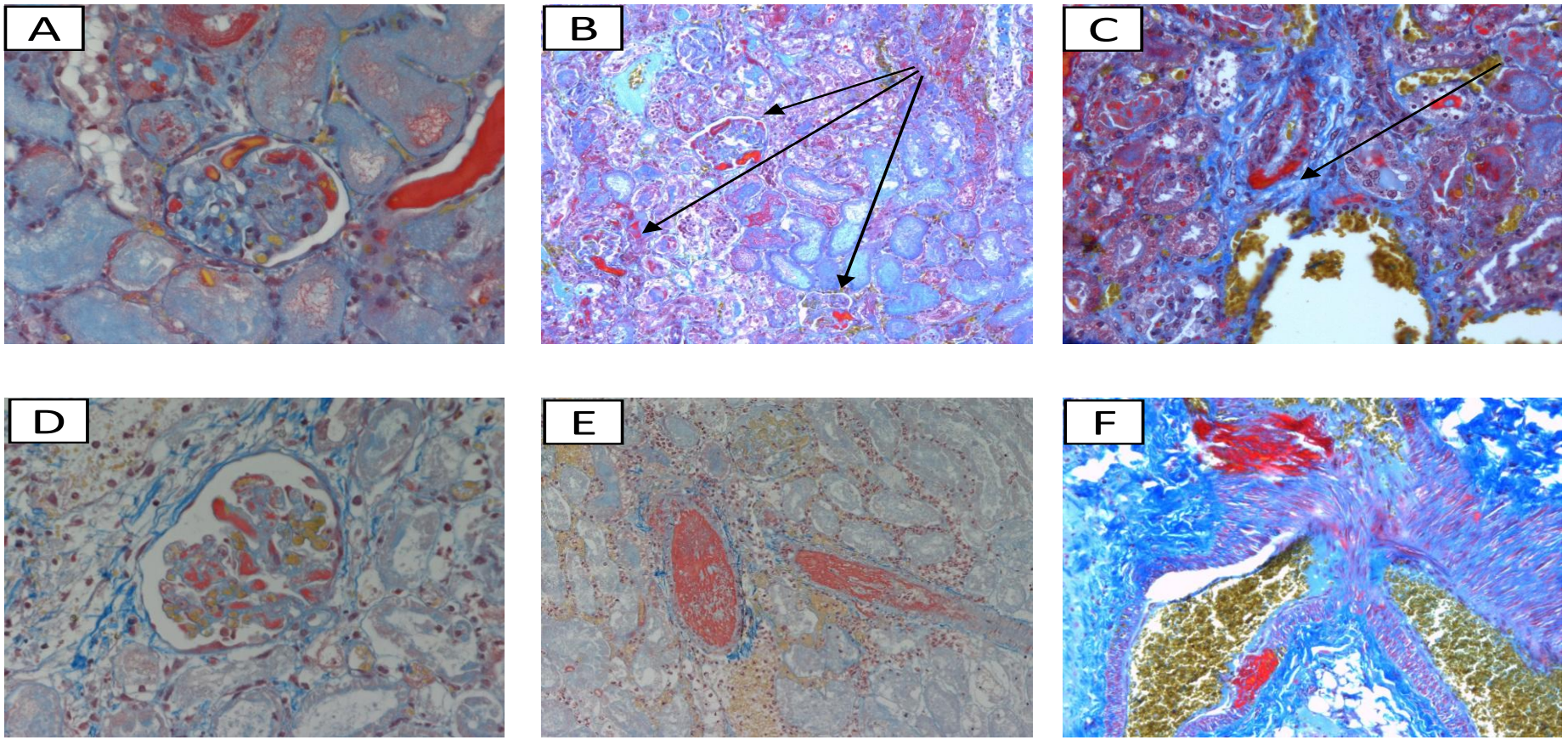


Figure 4.7: Donor kidneys treated with 80 µg/ml of APT070 or control APT154. A: Representative DA kidney allograft treated with APT070 (n=9, 3 sections/stain) taken from pre-sensitised Lewis recipients at day 1 post-transplantation showing glomerular thrombosis (MSB; x400). B:

Widespread thrombosis in the microvasculature with focal necrosis at day 2 post-transplantation (MSB; x100). C: Small vessel thrombosis at day 2 post-transplantation (MSB; x200). D: Picture of a representative DA kidney allograft treated with APT154 (n=4, 3 sections/stain) taken from pre-sensitised Lewis recipients at day 1 post-transplantation showing presence of glomerular thrombosis (MSB; x200). E: Infarction, extensive fibrinoid necrosis and haemorrhage at day 1 post-transplantation (MSB; x100). F: Large vessel thrombosis at day 2 post-transplantation (MSB; x200).

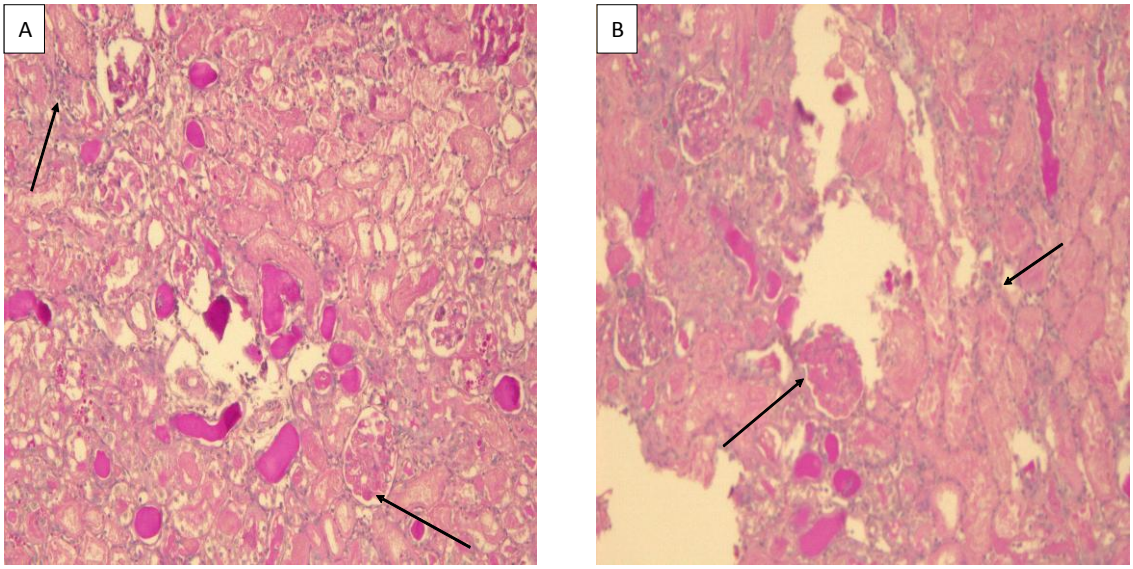


Figure 4.8: Micrographs of PAS staining taken at x100 of donor kidneys treated with 80 µg/ml of APT070 or control APT154. A: Histology of a representative DA kidney allograft treated with APT070 (n=9, 3 sections/stain) taken from a pre-sensitised Lewis recipient at day 1 post-transplantation showing focal infarction, glomerular thrombosis and leukocyte infiltration. B: Morphological features of a DA kidney allograft treated with APT154 (n=4, 3 sections/stain) taken from pre-sensitised Lewis recipients at day 1 post-transplantation showing widespread infarction and leukocyte infiltration. Lower arrow indicates a completely thrombotic glomerulus.

4.8 Discussion

The aims of the experiments described in this chapter were to: 1) characterize APT070 by performing an *in vitro* haemolytic assay to confirm its complement inhibitory activity; 2) investigate APT070 binding ability and distribution in the kidneys following intrarenal perfusion and; 3) determine the effects of the inhibitor in a rat model of HAR. This model represents the most severe type of rejection observed in sensitised recipients in the absence of a cross-matching protocol.

In vitro experiments confirmed the ability of APT070 to inhibit complement. In these experiments the concentration of APT070 required for 50% inhibition of haemolysis, mediated by activation of the classical pathway (IH50) was measured. In this assay, APT070 (IH50 ~1.5 nM) protects antibody sensitised sheep erythrocytes from lysis. The functional moiety of APT070, like CR1, is known to bind C3b and C4b, thus inactivating the classical C3 and C5 convertases. Thereby, it prevents the formation of membrane attack complex (MAC) on the surface of the sheep red blood cells, and so prevents red blood cell lysis by osmosis (Cole DS et al., 2003 **173**). Some inhibitory action was observed by the soluble control untagged SCR1-3 (APT154), however it was substantially less effective (IH50 ~85 nM). These data demonstrate the impact of the membrane tagging technology, maximizing the reagent efficacy.

One of the unique aspects of this work is that the therapeutic inhibitor is delivered to the donor kidney and not to the recipient. Systemic interference of complement is thus avoided and systemic complement as a host defense mechanism is maintained. Using the intrarenal delivery protocol it was confirmed that APT070 was able to distribute throughout the donor kidney in transplants carried out between syngeneic DA donors and recipients. Staining of tissue sections with an antibody against the SCR1-3 sequence of human CR1 showed that APT070 was still strongly detectible in the donor kidney at 0.5 hours post-transplantation, indicating that the inhibitor remained firmly bound. This is important considering the rapidity and site targeted by pre-formed antibody in this model. The primary target of AMR is the graft endothelium. It was evident that APT070 introduced by perfusion of the renal artery was localised to the microvasculature, both glomerular and peritubular. The larger vessel staining is difficult to interpret using the immunofluorescence due to non-specific staining. At 24 hours

post-transplantation, APT070 was focally located largely at the apical surface of the renal tubular epithelial showing that the inhibitor had been internalized.

In view of the functional and binding capabilities of APT070, it was considered to be an ideal inhibitor for targeting complement in this model of HAR. My experiments therefore went on to test the therapeutic inhibitor delivered by this means to the microvascular surface of the transplant to identify its potential and limitations in the most stringent conditions.

The initial dose of APT070 (40 μg/ml, 200μg) was based on previous findings in our lab, which showed a significant beneficial effect at this dose, in rat models of ischaemia-reperfusion injury and chronic rejection (Pratt J et al., 2003 **290**). However, APT070 at this dose had no major effect on graft survival or renal function compared to untagged inhibitory fragment of CR1 in my study of HAR. All animals died by 48 hours post-transplantation. Considering the severity of the injury in terms of speed of onset and degree of tissue damage in my model of HAR compared with the injury reported in the chronic rejection model, the concentration of APT070 was doubled to 80 μg/ml, 400μg). The APT070-treated group showed slightly better BUN values at 24 and 48 hours post-transplantation, compared with the control APT154 group, though the difference was not statistically significant. Histological analysis of both the APT070- and control-treated grafts revealed neutrophilic infiltration in the peritubular capillaries (PTC), tissue infarction and thrombosis. The apparent lack of effect of APT070 therapy to prolong graft survival was unexpected, due to the stringency with which APT070 inhibited complement-mediated lysis *in vitro* and its ability to localise on the microvascular surface of the donor organ following blood reperfusion (the acute phase of rejection).

Until recently, complement activation was considered to be a key mediator of HAR. Indeed, the endothelium of the microcirculation is the main target of HAR as alloantibody ligation to donor antigen expressed on the surface of the graft will almost certainly activate the complement system. This activation results in increased inflammation and loss of vascular integrity. In addition, initiation of complement activation has been associated with increased thrombosis (Colvin RB et al., 2005 **9**). Indeed, this model has a profound tendency for coagulation of the renal vasculature.

The failure of APT070 (at 40-80 μg/ml) to improve graft outcome in this model implies a complement-independent mechanism for initiating coagulation occurred. APT070 inhibits complement activation and accordingly prevents the main effector products of complement from being formed. Recent interest has been focused on the role of the activated endothelium itself, which can be stimulated directly by alloantibody alone, in the absence of complement. Antibody ligation on vascular surfaces activates the endothelial cells, which release proinflammatory molecules such as E-selectin, ICAM (CD106) and VCAM (CD54). This causes leukocyte adhesion and infiltration of inflammatory cells. These cells secrete cytokines such as TNF-α, IL-1 and IL-6 and upregulate endothelial expression of procoagulant tissue factor (TF). Most importantly, alloantibodies directly promote tissue injury by monocytes and NK-cells by antibody-dependent cellular cytotoxicity (ADCC) through their Fc-receptors. This effector mechanism is not only complement-independent but can be initiated by non-complement fixing antibodies (Lee CY et al., 2007 **68**); thus graft damage can occur in the absence of complement. In addition, it is of great importance, especially in this model, that antibody directly promotes exocytosis and release of vonWillebrand factor (vWf) resulting in proinflammatory (externalization of P-selectin and leukocyte rolling) (Sis B et al., 2010 **77**) and prothrombotic responses (initiation of coagulation and activation of cellular haemostasis) (Yamakuchi M et al., 2007 **307**). Furthermore, not only is it possible that coagulation is initiated independently of complement by such mechanisms, but it is also conceivable that coagulation might precede it. In this scenario, APT070 treatment of the graft might efficiently inhibit complement activation but due to coagulation taking place upstream of endothelial activation, it fails to prolong survival, leaving the donor organ subject to devastating thrombotic effects.

Finally, a recent body of work has revealed that coagulation, and more specifically thrombin, may be able to activate complement by a bypass mechanism. Consequently, APT070 would have been unable to inhibit activation completely used as single therapeutic reagent.

In conclusion, Mirococept is a potent inhibitor of the classical pathway of complement activation. Local use of this inhibitor is known to be active against all three complement-activation pathways, and it is of proven efficacy in the pathological setting of chronic rejection (Pratt J et al., 2003 **290**) and ischaemia/reperfusion (Patel H et al.,

2006 **291**). These models differ from the HAR model presented here, in that the HAR model is dominated by the effects of DSA and has a short ischaemia time (no appreciable cold ischaemia and approximately 25 minutes of warm ischaemia). The limited effect of APT070 in the present (admittedly extreme) model may suggest that early activation of the rat complement system by anti-donor antibodies is less important than the complement independent rapid endothelial activation triggered by antibodies resulting in the local release of proinflammatory and procoagulant mediators. To further investigate this idea, a therapeutic approach was developed for targeting coagulation in the HAR model, specifically focusing on thrombin, which could have pivotal role in orchestrating the damaging immune responses in this setting.

Chapter 5 – Impact of Therapeutic Coagulation Inhibitor PTL006 on HAR

5.1 Introduction

The lack of therapeutic benefit with the complement regulator APT070 and large amount of residual thrombotic microangiopathy prompted an investigation into the effect of coagulation blockade in this model of extreme HAR. My hypothesis was that endothelial cell protection against thrombogenesis would ameliorate the consequences of HAR initiated by preformed DSA in the transplant recipient. The focus of the present chapter is a prototype membrane-targeted antithrombotic agent PTL006, developed in our laboratory. The effect of donor organ pre-treatment with this agent on HAR was investigated.

PTL006 is a powerful anticoagulant which incorporates the hirulog like (HLL) peptide isolated from hirudin and a membrane localizing tail. Its anticoagulant ability is attributed to the fact that bivalent interactions with thrombin inhibit the enzyme and consequently impair a variety of biological responses. In the hyperimmune model, therapeutic intervention with PTL006 could target thrombin's procoagulant and proinflammatory action against the transplanted kidney to improve outcome.

In a similar fashion to APT070, PTL006 is an engineered therapeutic (though an anticoagulant) which directly binds to the graft endothelium via its tail. PTL006 is the first in a series of cytotopic anticoagulant reagents and its tail differs from the one possessed by APT070. PTL006 binds to the membrane, forming hydrophobic interactions with the phospholipid bilayer through a PEGylated modified tag. Characterization of this reagent was a starting point for my work, which then went on to evaluate its effect in the hyperimmune model. Using the intrarenal perfusion protocol, a series of syngeneic and allogeneic transplants were performed to confirm the appropriate working concentration of the tailed antithrombin, to examine its ability to bind to cells and determine whether administration by this route induced systemic anticoagulation. The anticoagulant used for the *in vivo* experiments was PTL006 and then FAM-labelled PTL006 (PTL006-FAM) for immunohistochemistry. This novel set of agents were generated and kindly provided by Dr R Smith (KCL).

After determining an appropriate dose, PTL006 was perfused via the renal artery into DA donor kidneys and the PTL006-treated kidneys were transplanted into pre-sensitised Lewis recipients. The allotransplantation model used in these studies was characterized by thrombotic microangiopathy, making it an ideal model for dissecting the contribution of coagulation to graft outcome. This approach is unusual as catastrophic coagulation in humoral immune responses has mainly been addressed using xenotransplantation models. For instance, in a cardiac xenotransplantation model, mouse hearts were transplanted into rat recipients, whose fibrinogen had been depleted using ANCROD, a snake venom. In this study acute xenograft-mediated rejection was delayed by treatment (Chen D et al., 2006 **308**). More relevant to this study, hearts from transgenic mice expressing the anticoagulant protein hirudin, expressed on the endothelium of the xenograft, were transplanted into rats. These experiments demonstrated that inhibition of thrombin resulted in elimination of xenograft rejection (Chen D et al., 2004 **309**). Based on these findings, my experiments addressed the hypothesis that intrarenal delivery of anticoagulant PTL006 could reduce antibody-mediated coagulopathy and inflammation. Furthermore, the model I developed (Chapter 3) uses inbred rats in an allotransplantation setting rather than transgenic organs in xenotransplantation. In this regard, the model described here may be considered more clinically relevant.

5.2 Experimental Design

An initial series of transplantation experiments was performed to determine a suitable dose of PTL006, i.e. one that would enable anticoagulation but avoid system anticoagulation and potential bleeding during transplant surgery. Syngeneic transplantation was carried out between DA donors and recipients, using a perfusion volume of 5ml and concentration of PTL006 at 5 or 1 μM. Allogeneic transplant experiments were then performed between DA donors and naïve (i.e. non-sensitised) Lewis recipients using the same dosing regimen. During the transplant surgery, any bleeding at the site of the anastomosis after release of the microvascular clips was scored. Controls included DA kidneys treated with vehicle Soltran solution alone. Subsequent transplant experiments between DA donors and hyperimmune Lewis recipients used a perfusion volume of 5ml to deliver the PTL006 intra-renally at concentrations of 5, 1, 0.1 or 0.01 μM. The same controls and intra-graft delivery perfusion protocol were used (as described in Chapter 3). A smaller set of experiments

involved transplantation of DA kidneys treated with 2 μM PTL006 (concentration based on *in vitro* data, Melchionna T, unpublished data) into the sensitised Lewis recipients.

To establish the distribution of retained anticoagulant in the donor kidney, DA kidneys were perfused with FAM-labelled PTL006 and transplanted into syngeneic recipients. The grafts were removed at 0.5 (n=1) and 24 (n=1) hours post-transplantation and frozen kidney sections were prepared and examined by fluorescence microscopy. Tissue from kidneys perfused with Soltran solution alone and harvested at the same time points served as negative controls (n=1 per time point).

For the transplants involving hyperimmune recipients, two treatment groups were studied. The first group involved PTL006 given alone (to the donor kidney); the second group involved a combination of PTL006 and APT070 given to the donor organ. The purpose was to look for any additive effects of coagulation and complement inhibition on graft survival. As before, graft survival, renal function (BUN) and graft morphology were used to assess treatment outcomes

5.3 *In vivo* Dose-finding study of Coagulation Inhibitor PTL006

PTL006 is the first of a series of novel cytotopic anticoagulants which vary in the nature of their membrane-interactive tail. This derivative contains polyethylene glycol (PEG) and it has been shown to be a powerful anticoagulant *in vitro* (Melchionna T et al., unpublished data). My transplant experiments describe the first *in vivo* use of PTL006, and are listed in tables 5.1 and 5.2 below.

For the first set of experiments, syngeneic DA donor organs were treated with Soltran containing PTL006 using an intrarenal infusion rate of 1ml/min over 5 minutes. The aim was to exclude any toxicity to the graft and any tendency to bleed induced in the recipient by PTL006. These recipients recovered quickly and survived in good health for the entire length of the experiment due to the functioning graft (which was life-supporting as bilateral nephrectomy was performed the day of the transplantation). The doses used in these initial experiments were 5 ml at 5 μM (n=2) and 1 μM (n=2) (based on *in vitro* results, Melchionna T, unpublished data). Recipient rats showed no sign of distress and were killed at day 10 post transplantation (Table 5.1). However, post operative bleeding (heavy but manageable) was noted at the site of the anastomosis in recipients of PTL006-treated grafts. In these animals, bleeding was prolonged and required repetitive application of pressure and continuous swabbing with surgicel. This was in contrast to recipients of Soltran-treated control isografts (n=2), where no bleeding was observed. In the second group of transplants, where naive Lewis recipients were transplanted with DA allograft treated with the same doses of PTL006, prolonged bleeding was also noted. This occurred for both the 5 μM and 1 μM PTL006 treatment groups. Again, no bleeding was observed for the Soltran-treated donor kidneys (Table 5.1).

Apart from the local bleeding, all recipients of PTL006-treated kidneys recovered well, with no apparent compromise of survival in syngeneic transplants, or in allogeneic transplants in the non sensitised group of recipients. In the latter group, the graft survival data correspond to the figures for control allogeneic DA to naïve Lewis kidney transplants described in Chapter 3 (MST 5 days). A summary of all the transplants performed in non-sensitised recipients is shown (Table 5.1).

DONOR	RECIPIENT	DOSE (μM)	n	BLEEDING SCORE	SURVIVAL
DA	DA	5	2	++	end at d10
DA	DA	1	2	++	end at d10
DA	DA	SOLTRAN	2	0	end at d10
DA	Lewis	5	2	++	d7
DA	Lewis	1	2	++	d7
DA	Lewis	SOLTRAN	2	+	d5

+ + heavy but manageable, + normal bleeding,

Table 5.1: Summary of syngeneic and allogeneic kidney transplants performed between DA donors and DA or naïve Lewis recipients. Grafts perfused with PTL006 (1 μM or 5 μM in 5ml). Bleeding scores at the anastomosis site are shown. Bleeding did not affect graft survival.

As described previously, PTL006 caused abnormally heavy bleeding upon release of the microsurgical clips, however it was successfully controlled.

The next set of experiments explored the effect of PTL006 in presensitised Lewis recipients of DA donor kidneys using the same treatment protocol as above. This used the same dose of PTL006 as in the previous experiments, with naïve recipients. In this case, where sensitised recipients were used, severe and uncontrolled bleeding occurred at the site of the anastomosis and all efforts to rescue the grafts failed, resulting in all grafts becoming ischaemic. Disparity of vessel sizes was thought to be pivotal to this problem and unavoidable for a number of reasons.

Experiments were carried out to find the minimum dose for efficacy in sensitised recipients. These revealed that PTL006 at 5 (n=1), 1 (n=2) and 0.1 (n=2) μM also resulted in severe bleeding. However further dose reduction of PTL006 to 0.01 μM (n=3) avoided this problem, so that the rats survived the transplant procedure at this dose of treatment. Although no extension of graft survival occurred in the sensitised rats (MST 2 days post-transplantation) compared to treatment with Soltran (MST 2 days), this experiment confirmed that the bleeding tendency in PTL006-treated kidneys was dose-related. In order to deal with the possibility that not all of the infused material was bound to the donor kidney endothelium, a change in the perfusion protocol was employed. Two extra steps were added: at the end of the 5 minute intrarenal perfusion procedure, before excising the donor kidney for transplantation to the recipient, a dwell-

time of 10 minutes was introduced to allow the reagent to bind to the endothelium and a second flush of the donor organ was introduced with 5 ml of Soltran solution (at 1 min/ml rate for 5 minutes) to remove any unbound material. In this modified perfusion protocol, the optimal concentration of PTL006 was 2 μM (a compromise between the 5 and 1 μM doses). For the first time in the pre-sensitised recipient, treatment of the donor organ with a single cytotopic reagent prolonged the graft survival from MST day 2 to day 5 in (n=2). The treatment protocol for future experiments in sensitised recipients was based on these conditions. A summary of the transplants performed for this set of experiments in hyperimmune recipients is shown in table 5.2.

DONOR	RECIPIENT	DOSE (μM)	n	BLEEDING SCORE	SURVIVAL
DA	Lewis	5	1	+++	no
DA	Lewis	1	2	+++	no
DA	Lewis	0.1	2	+++	no
DA	Lewis	0.01	3	+	d2
DA	Lewis	SOLTRAN	2	+	d2
DA	**Lewis**	**2**	**2**	**+**	**d5**

+ + + *severe bleeding,* + *normal bleeding*

Table 5.2: Summary of kidney transplants performed between DA donors and pre-sensitised Lewis recipients. Perfusion of the donor organ with PTL006 at 5, 1 and 0.1 μM resulted in severe bleeding and the recipients did not survive. At 0.01 μM of PTL006 the bleeding was normal, but the anticoagulant failed to extend survival. In the bottom-row (highlighted), two changes were made: the infusion dose of PTL006 was 2 μM; and a dwell-time of 10 minutes was introduced before flushing out the kidney with Soltran. **This eliminated the bleeding tendency and enabled a higher dose of PTL006 (2 μM) to be used. A higher MST of 5 days was achieved.**

5.4 Tissue Distribution of PTL006 in the Kidney Transplant

The tissue distribution of the anticoagulant was determined by perfusion of DA kidneys with 2μM PTL006 directly labeled with FAM (PTL006-FAM), a FITC-like fluorochrome. PTL006 becomes inactive (that is, the therapeutic functional activity is lost when labelled with FAM but the tail binding is retained), therefore, this reagent was used for purely distribution purposes. PTL006-FAM-treated kidneys were transplanted into syngeneic recipients and Soltran-treated kidneys were used as controls. Following the modified perfusion protocol incorporating the extra dwell-time and flush, kidneys were transplanted and then removed at 0.5 (n=1) and 24 (n=1) hours post-transplantation. The kidneys were snap frozen for storage in liquid nitrogen and tissue sections later analysed by fluorescence microscope. As shown in figure 5.1 PTL006-FAM was clearly retained on the glomerular capillary wall at 0.5 hours post transplantation. At 24 hours post transplantation, fluorochrome-labelled anticoagulant was strongly detected on both distal and proximal tubular epithelium, but no longer on the glomerular capillary tuft. Control Soltran-treated kidneys removed at both time points showed no positive staining (n=1 per time point).

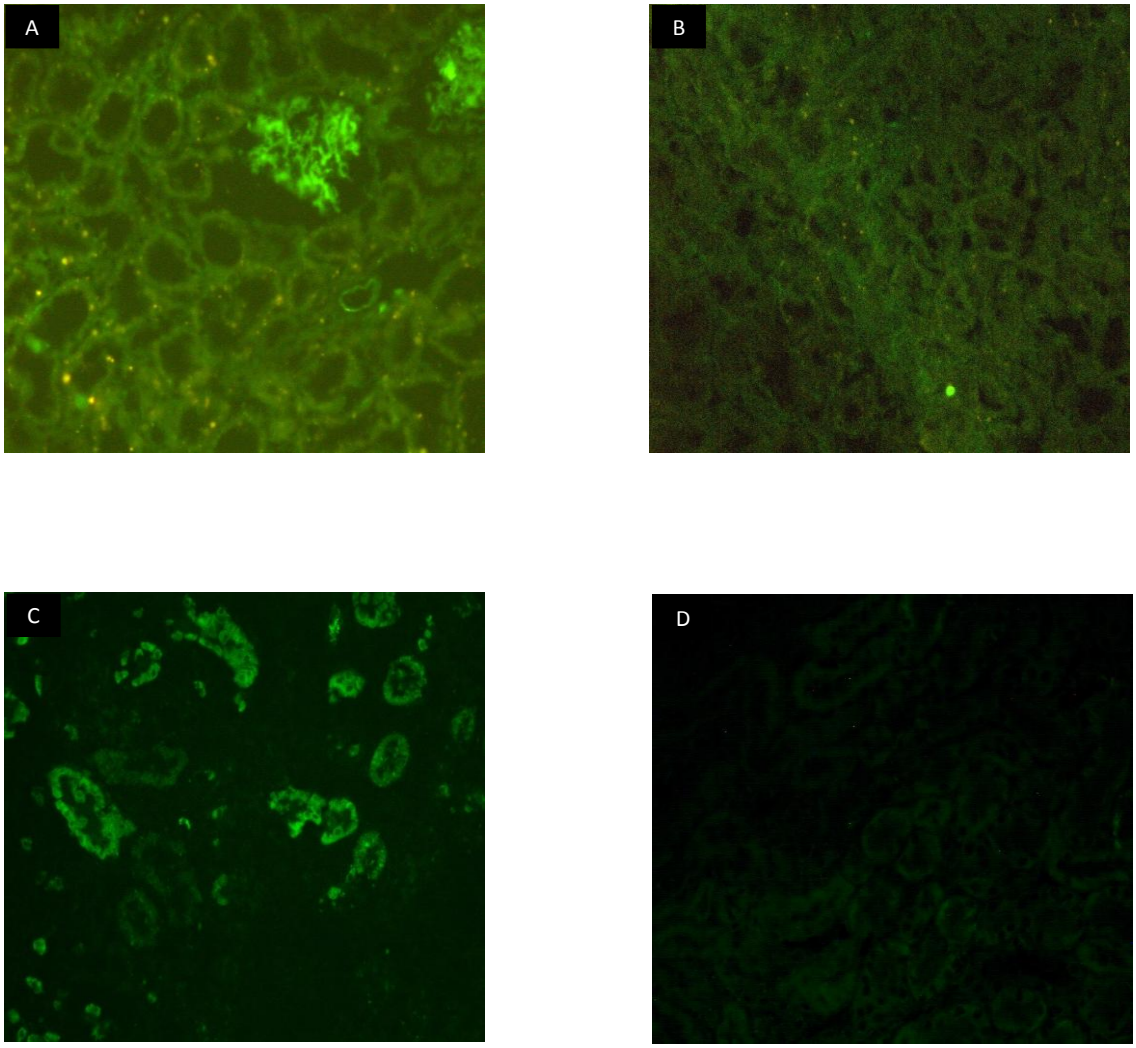


Figure 5.1: Immunofluorescent detection of PTL006-FAM in DA donor graft removed from syngeneic recipients. A: PTL006-FAM observed in the glomeruli at 0.5 hours post-transplantation. B: No detection in DA donor Soltran-treated graft at 0.5 hours post-transplantation. C: Strong PTL006-FAM deposition on distal and proximal tubules at 24 hours post-transplantation. D: No detection in DA donor graft treated with Soltran perfusion solution at 24 hours post-transplantation. Representative images were taken at x200.

5.5 Graft Survival in Pre-sensitised Lewis Recipients of PTL006-treated DA donor Kidneys

Employing the modified perfusion protocol, DA donor kidneys were treated with PTL006 or control Soltran solution alone before transplantation into presensitised Lewis recipients. Following transplantation, the effect of the coagulation inhibitor on graft survival was observed. The results showed that DA donor kidneys pre-treated with 2 μM PTL006 had significant prolongation of survival in the hyperimmune Lewis recipient (MST 4.6 days), compared to Soltran perfused kidneys (MST 1.8 days) (Figure 5.2).

In the PTL006-treated group (n=8) there was 100% graft survival at 24 hours post transplantation compared to the Soltran-treated group in which 16.6% of grafts had failed (1 of 6). The remaining grafts of the control group (83.3%) had failed by 48 hours post transplantation (5 of 6). In contrast, only 12.5% of PTL006-treated grafts had failed by 48 hours post transplantation (1 of 8), the average MST for survival of pre-sensitised recipients in this hyperimmune model. 50% of animals that received PTL006-treated kidneys (4 of 8), had prolonged graft survival to day 5 post transplantation. The survival of the remaining 3 animals was extended to day 3, day 4 and day 6 (1 of 8 at each time point) (Figure 5.2). Graft loss was defined as high serum BUN, death of the recipient, or when the animal had to be killed due to obvious distress, which ever came earliest.

Thus, a consistent increase in graft survival corresponded to the application of PTL006, (2 μM in 5 ml) to donor kidney despite the presence of high titre antidonor antibodies, which in the control group led to predictably aggressive graft loss. Given the severity of the unprotected injury, survival-increase by several days was a significant feat, as was the ability to perform transplant surgery with no bleeding complications.

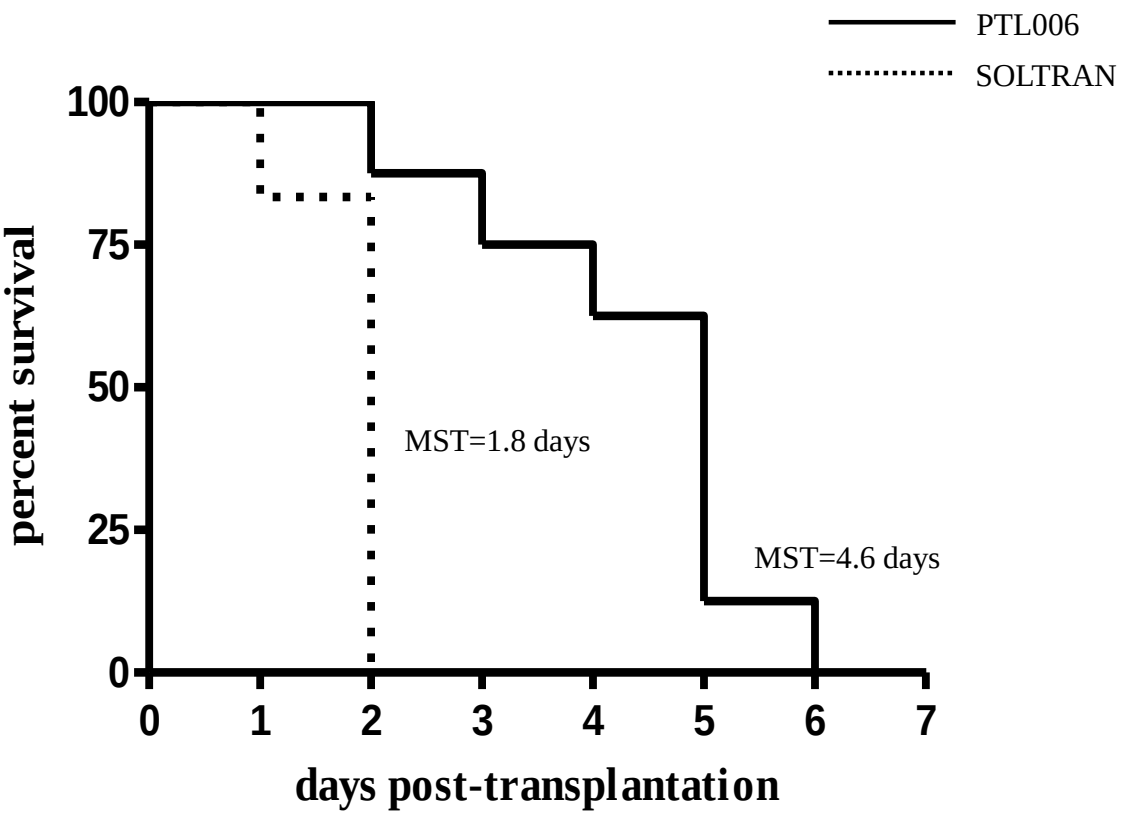


Figure 5.2: Graft survival curve following transplantation of DA kidneys treated with PTL006 or Soltran alone into pre-sensitised Lewis recipients. PTL006 treatment of donor organs (5 ml at 2 μM) transplanted into pre-sensitised rats (n=8) achieved significant prolongation of survival compared to the Soltran-treated control group (n=6) (p=0.0016).

5.6 Renal Function in Pre-sensitised Lewis Recipients of PTL006-treated Kidneys from DA donors

The renal function of graft recipients in the experiment described in section 5.5 was monitored daily until graft failure occurred. The BUN levels for sensitised recipients that received DA kidney pre-treated with 5 ml of 2 μM of PTL006 were lower and increased more gradually compared to that of recipients of Soltran-treated transplants (Figure 5.3A). Given the severity of the vascular injury by 24 hours post-transplantation in the untreated HAR model, it is remarkable that the renal function of the PTL006-treated group was almost in the normal range (BUN<10mmol/L) at this time-point, whereas the BUN in the Soltran-treated group was significantly impaired with BUN approximately 30 mmol/L (Figure 5.3B).

So, while treatment with the complement regulator APT070 had provided little or no benefit (Chapter 4), the anti-thrombin protocol developed for PTL006 appeared promising, since a safe dose of PTL006 (2 μM) prevented sudden loss of graft function despite the high titres of pathogenic antibody against the graft.

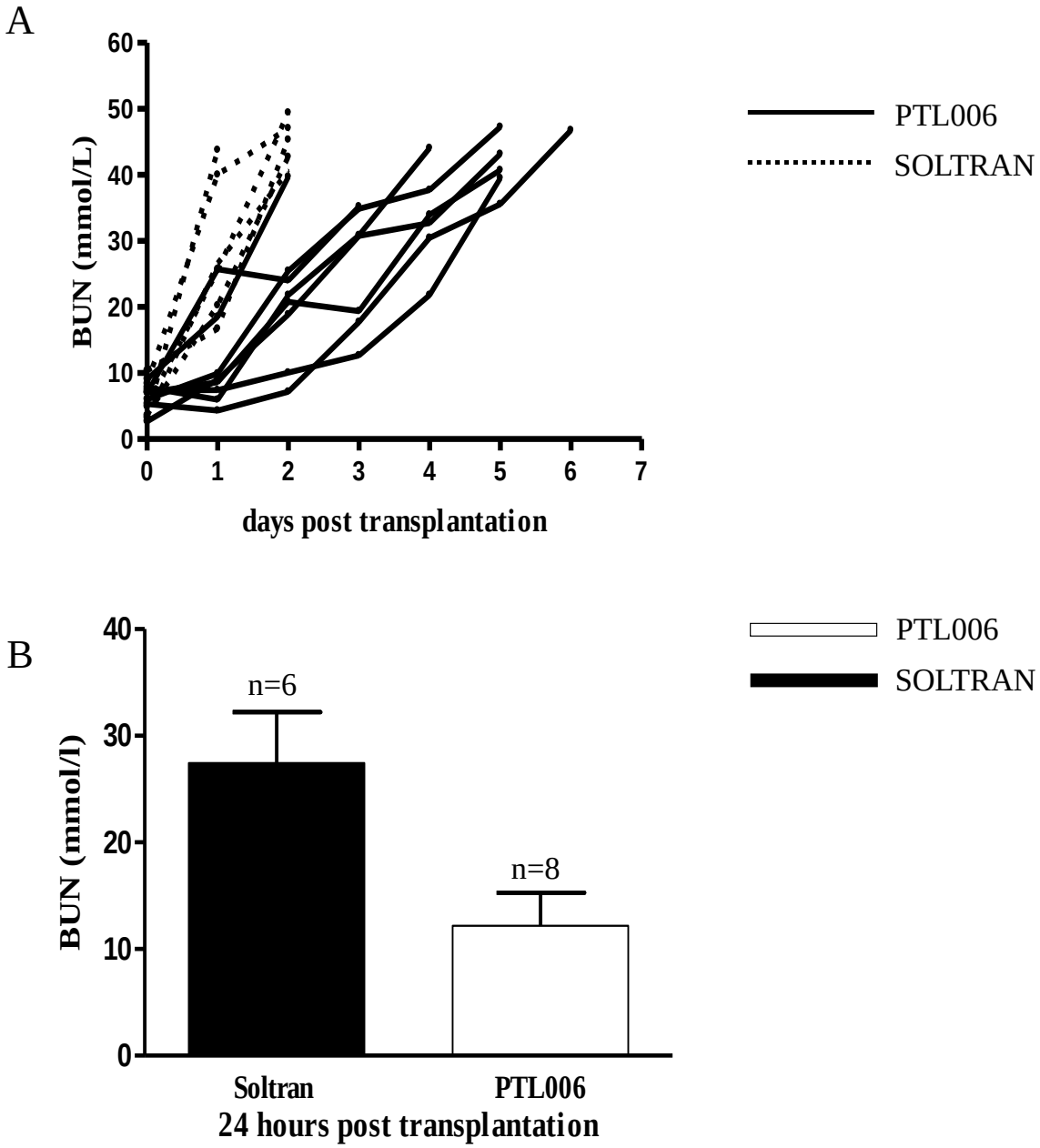


Figure 5.3: Renal function (BUN) post-transplantation following treatment of the donor organ with PTL006 (5 ml at 2 μM) or Soltran control. A: PTL006 treatment of DA donor organ slowed the decline of renal function in hyperimmune Lewis recipients. In contrast, there was immediate dysfunction in recipients of Soltran-treated grafts. B: BUN at 24 hours post-transplantation (p=0.0426) showing good immediate preservation in renal function for the PTL006-treated group.

5.7 Graft Pathology in Pre-sensitised Lewis Recipients of PTL006-treated Kidneys from DA donors

As mentioned, MST for PTL006-treated grafts transplanted into hyperimmune recipients was 4.6 days post-transplantation, compared to 2 days for controls. Histological examination of protected grafts was carried out at day 5 (n=4) and compared to those of Soltran-treated grafts (n=5) rejected at day 2 post-transplantation (MST was 1.8 days post-transplantation).

Histology of the treated kidneys showed signs of antibody-mediated rejection, namely extensive fibrinoid necrosis and glomerular thrombosis with the presence of inflammatory cells on the peritubular capillaries. In addition, there was evidence of cell-mediated rejection, i.e. diffuse lymphocytic infiltrate with invasion of the renal tubular epithelium and the glomerular and arterial endothelium (Figure 5.4). In contrast, the Soltran-treated kidneys (at day 2 post-transplantation) revealed only antibody-mediated rejection and absence of infiltrating T-cells (Figure 5.5).

This assessment of graft morphology (at these 2 different time points) revealed that a single intrarenal dose of PTL006 was able to slow the rejection process from an aggressive, instant antibody-mediated rejection and convert it into a less vigorous acute antibody and cellular rejection. This could have important clinical implications as the latter may be more treatable or preventable with the addition of conventional immunosuppressive agents.

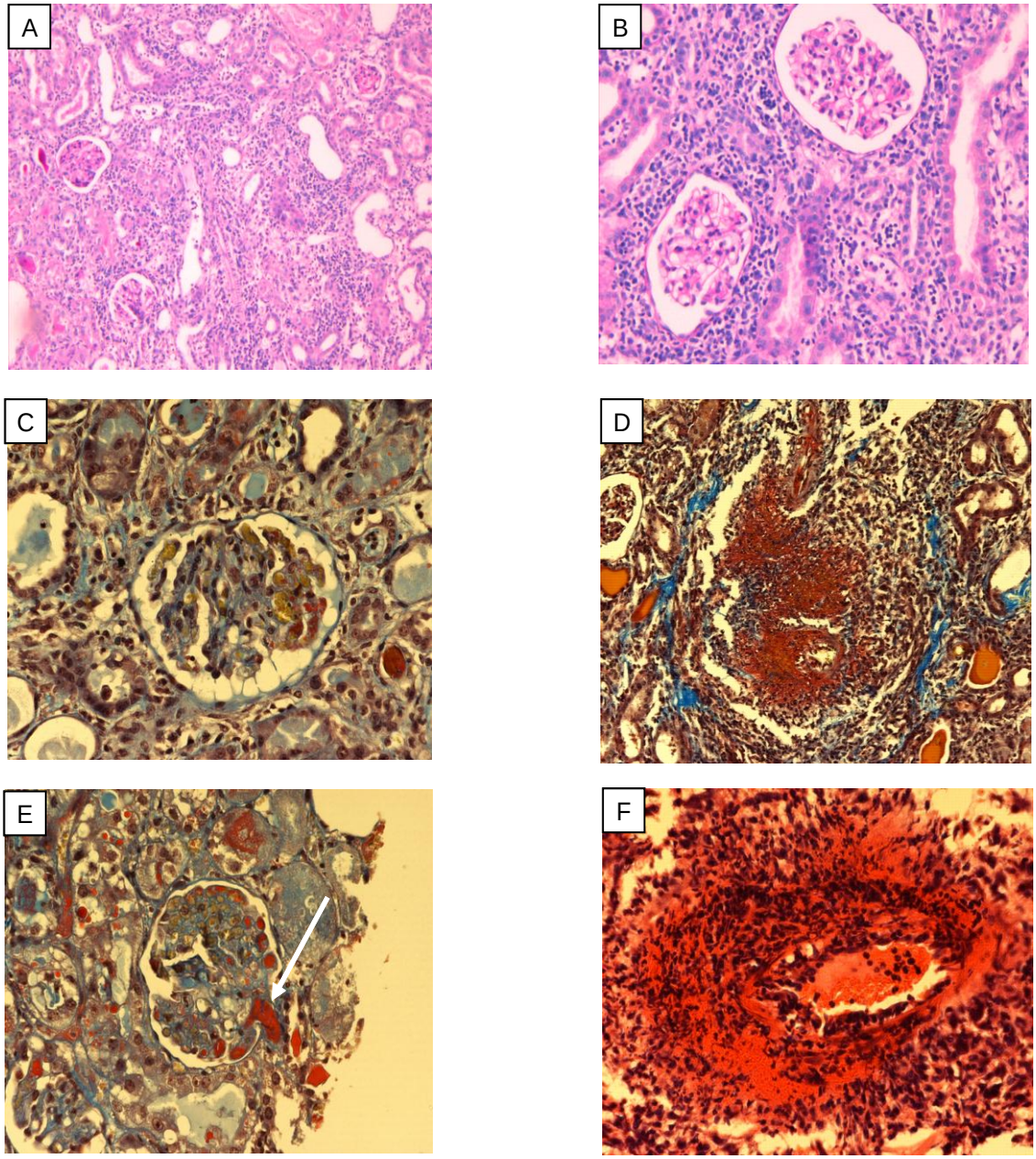


Figure 5.4: Histology of donor kidneys treated with PTL006. A: Representative tissue from a DA kidney allograft treated with PTL006 (n=4), taken from a pre-sensitised Lewis recipient at day 5 post-transplantation showing widespread lymphocytic tubulitis (H&E; x100). B: Tubulitis and glomerulitis (H&E; x200). C: Thrombosed glomerulus and surrounding tubules with characteristic lymphocytic infiltration (MSB; x400). D: Fibrinoid necrosis (MSB; x200). E: Fibrin deposition in a glomerulus (MSB; x400). F: Severe arterial fibrinoid necrosis (H&E; x400).

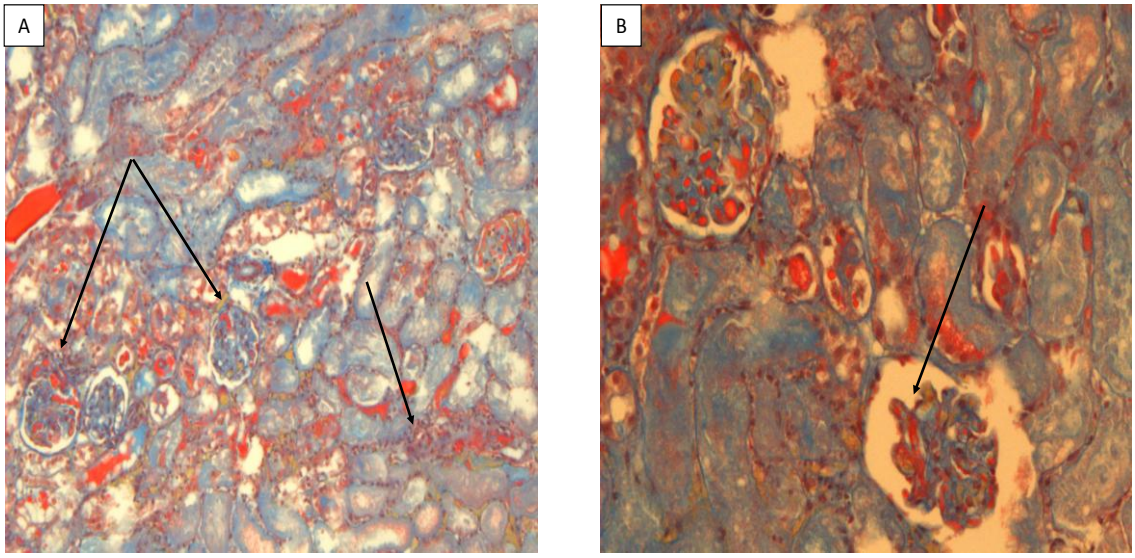


Figure 5.5: Control donor kidneys treated with Soltran alone. A: Histology of representative DA kidney allograft treated with Soltran (n=5) and removed from a pre-sensitised Lewis recipient at day 2 post-transplantation showing extensive thromboangiopathy (arrows), neutrophilic and monocytic infiltration on the PTC and general infarction (MSB; x100). B: Thrombotic glomeruli (arrow) and surrounding necrotic tubules (MSB; x200). The abnormalities are typical of severe AMR.

5.8 Results of Combined PTL006/APT070 Treatment of donor Kidney in Sensitised Recipients

After investigating the effects of the coagulation inhibitor PTL006 alone, an additional set of transplants was carried out. In this group, hyperimmune Lewis rats received DA kidneys that had been treated simultaneously with a mixture of PTL006 and APT070, to investigate potential synergy of coagulation and complement blockade on graft survival. DA donor kidneys pre-treated with 2 μM of PTL006 and 80 μg/ml of APT070 resulted in a significant prolongation of survival of the hyperimmune Lewis recipients (MST 5.5), compared to the recipients that received Soltran perfused kidneys (MST 1.8 days).

In the PTL006/APT070-treated group (n=8) no graft loss occurred within 48 hours of transplantation, whereas in the Soltran-treated group 100% of the kidneys were rejected by 48 hours post-transplantation. The remaining animals of the control-treated group (83.3%) had to be culled at 48 hours post transplantation (5 of 6) with no survival beyond this time point. In contrast, 25% of the PTL006/APT070-treated group had a prolonged graft survival to day 4 post-transplantation (2 of 8) and the remaining grafts were lost by day 7 (Figure 5.6).

Thus, simultaneous inhibition of coagulation and complement following a single treatment of membrane-localizing inhibitors, PTL006 (5 ml of 2 μM) and APT070 (80 μg/ml) achieved significant prolongation of survival compared to control treatment of the donor organ.

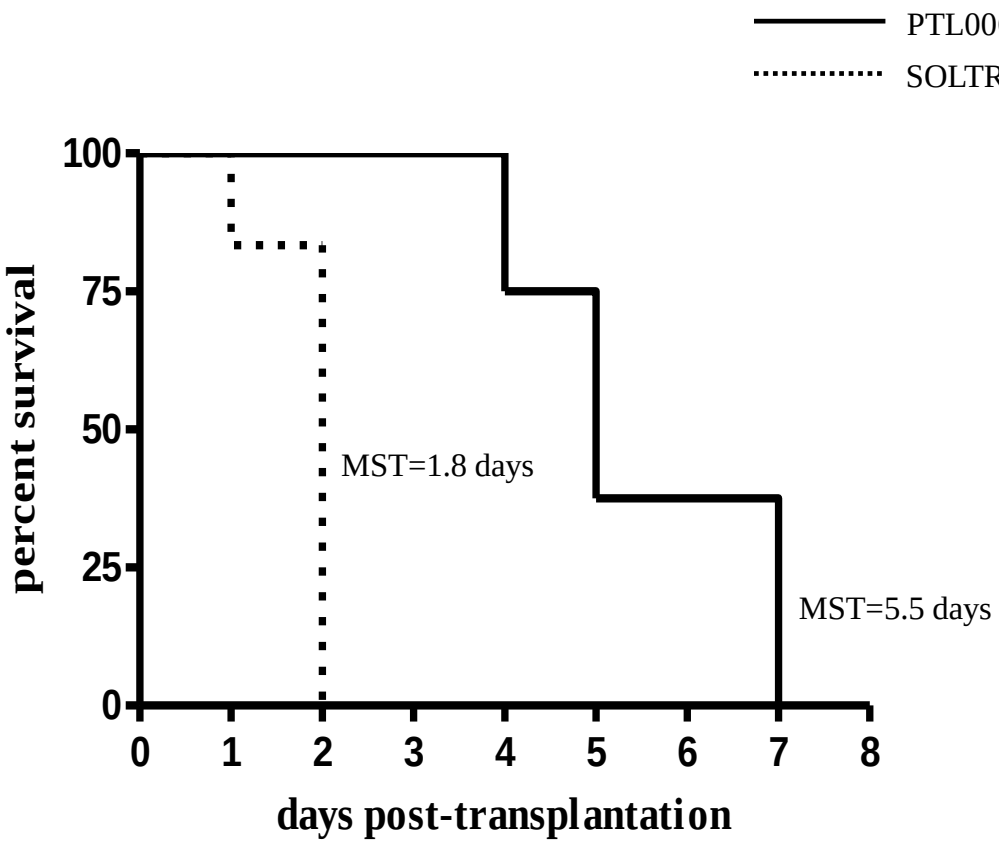


Figure 5.6: Graft survival curve following transplantation of DA kidneys treated with PTL006/APT070 into pre-sensitised Lewis recipients. The combined treatment of donor organs with 5 ml of 2 μM PTL006 and 80 μg/ml APT070 (n=8) achieved significant prolongation of survival, compared with a Soltran-treatment group (n=6) (p=0.0003).

5.9 Renal Function in Pre-sensitised Lewis Recipients of DA Kidneys treated with a combination of PTL006 and APT070

The BUN levels for the experimental groups described in section 5.8 were found to correspond with the graft survival data. The BUN levels showed a more gradual renal decline (BUN increase) over time, compared to recipients with Soltran-treated donor organs (Figure 5.7 A). There seemed to be a clear (but not significant) difference in the level of protection provided by the combination therapy at 24 hours post transplantation. BUN in the control group was approximately 30 mmol/L whereas in the PTL006/APT070-treated group, the mean BUN was approximately 15mmol/L (Figure 5.7 B).

These data indicate that pre-treatment with PTL006 and APT070 protected the donor organ against instant loss of renal function after transplantation into a hyperimmune recipient. This was apparent at 24 hours post-transplantation after the onset of HAR. It is not yet clear if this combination is more effective than PTL006 alone, since no direct comparison between the single and combined treatment was feasible in this experiment.

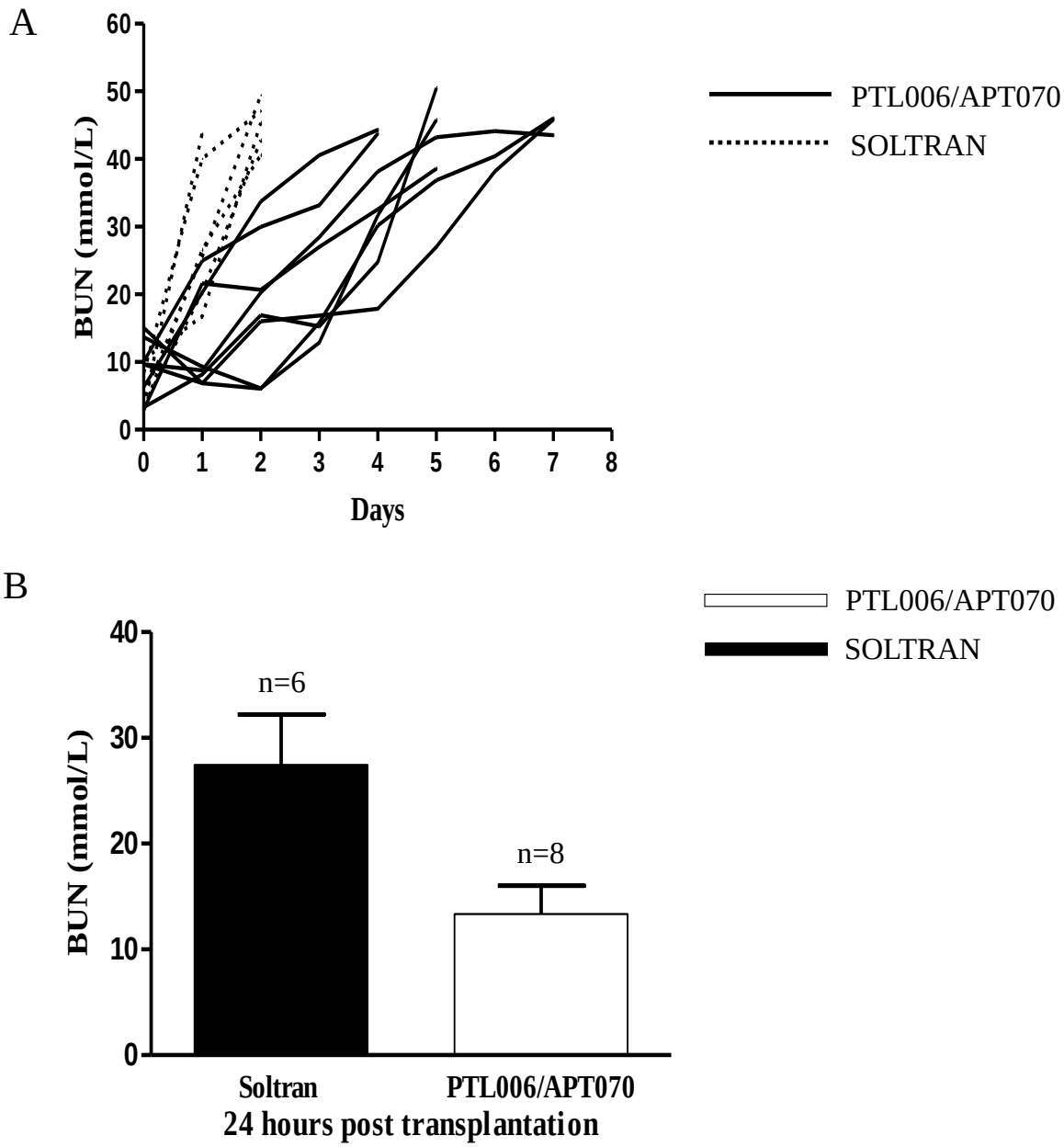


Figure 5.7: Renal function with combined PTL006/APT070 intervention. A: Combination treatment of the DA donor organ with 5 ml of 2 μM of PTL006 and 80 μg/ml of APT070, improved initial renal function of the kidneys in transplanted hyperimmune Lewis recipients with a relatively gradual impairment observed over time. This was in contrast to the immediate dysfunction of the kidneys in the Soltran-treated group. B: A difference in BUN between the PTL006/APT070-treated group and Soltran group at 24 hours post transplantation was observed but it was not significant (p=0.0593). Data shown was generated simultaneously to that shown in Figure 5.3. The same control group is shown in each graph for comparison.

5.10 Graft Pathology of PTL006/APT070-treated donor organs transplanted into Pre-sensitised Lewis Recipients

An MST of 5.5 days was achieved in donor organs pre-treated with a combination of PTL006 and APT070, following transplantation into sensitised Lewis rats. This was in contrast to MST 2 days for Soltran-treated controls. Histological data was obtained for kidneys rejected from three animals at day 5 post-transplantation and from three animals at day 7 post-transplantation.

Essentially, the changes produced by the treatment combination were similar to those described with PTL006 treatment alone, earlier in this chapter. Kidneys assessed at day 5 and day 7 post-transplantation showed signs of humoral as well as cellular rejection (Figure 5.8). This mixed pattern of rejection involved significant lymphocytic infiltration and invasion of the basement membranes of the renal tubules, causing tissue disruption. In parallel there was ongoing acute AMR with monocytes in the glomerular and peritubular capillaries. Fibrinoid necrosis was present in all grafts. This is in contrast to the Soltran-treated kidneys, which at day 2 post-transplantation exhibited features of pure AMR, with a clear absence of lymphocytes (Figure 5.5).

Thus, combined intrarenal delivery of PTL006 and APT070, morphologically, was able to modify the rejection pattern from an early aggressive, primarily vascular type of rejection, and convert it into to a predominant pattern of cellular rejection alongside milder vascular changes, which may have important implications for therapy.

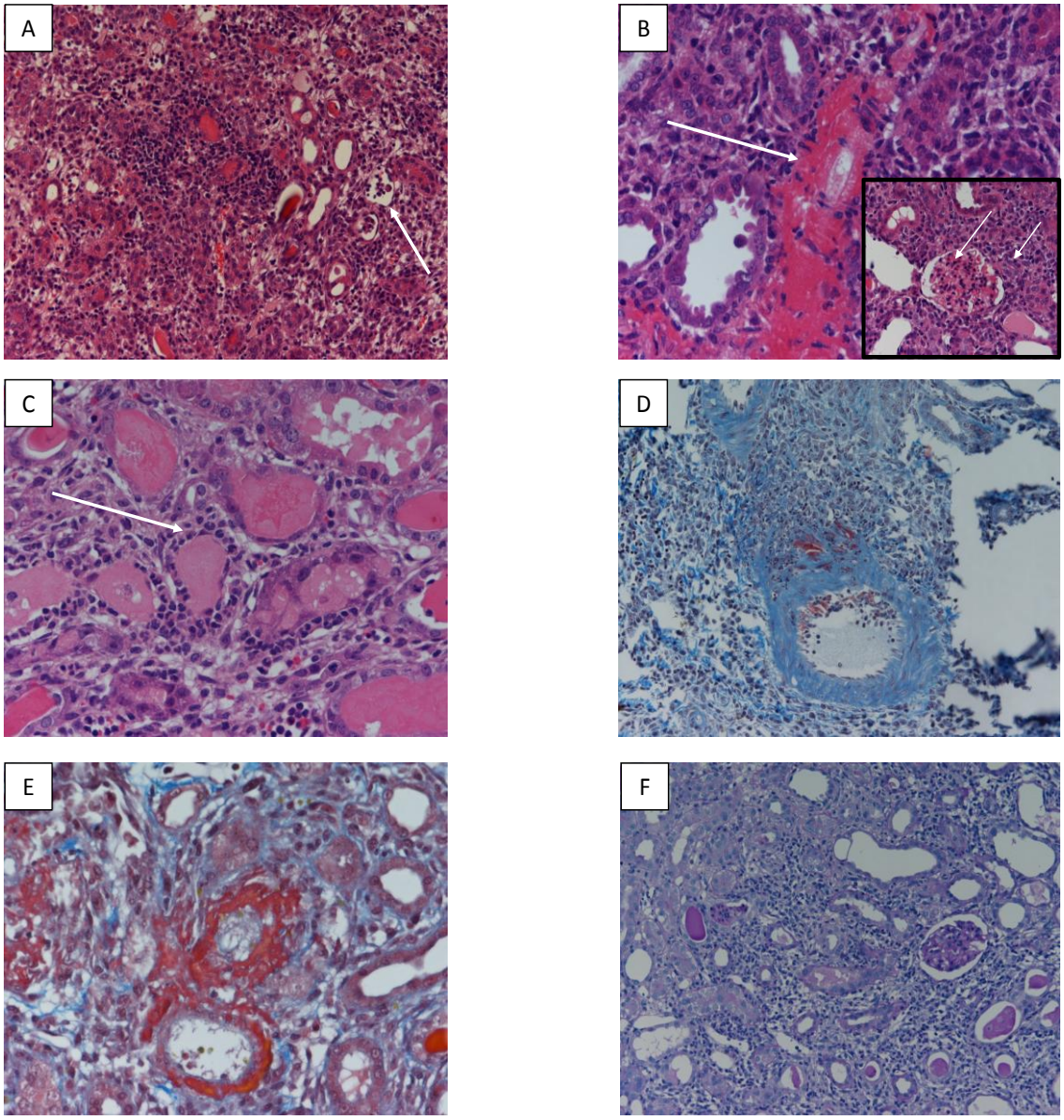


Figure 5.8: Histology of DA donor kidneys treated with PTL006 and APT070 following transplantation into pre-sensitised Lewis rats. A-D: Representative findings from allografts rejected at day 5 post-transplantation (n=3). A: Area of extensive lymphocytic tubular infiltration and peritubular capillaritis (H&E; x100). B: Fibrinoid necrosis (H&E; x400). Inset shows glomerulitis and tubulitis (H&E; x400). C: Lymphocytes entering the tubular basement membranes (H&E; x400). D: Arterial fibrinoid necrosis (MSB; x200). E and F: Representative findings from treated kidney allografts rejected at day 7 post-transplantation (n=3). E: Fibrinoid necrosis (MSB; x400). F: Lymphocytic infiltration (PAS; x100).

5.11 Discussion

The main objective of the experiments described in this chapter was to characterize *in vivo* the novel cytotopic coagulation inhibitor PTL006. More specifically, it aimed to determine a safe working concentration for intrarenal delivery, confirm binding of PTL006 within the kidney and determine its distribution. A final aim, once defining the desired characteristics, was to test this thrombin inhibitor in the model of HAR.

The dose-ranging study was performed using a series of syngeneic and allogeneic transplants between DA donors and DA or naïve Lewis recipients. The syngeneic transplants, in which the DA kidney was perfused with varying concentrations of PTL006, showed that PTL006 was not directly pathogenic to the kidneys and graft survival was unimpaired, compared to recipients of Soltran-treated control grafts. Two different concentrations of the inhibitor were tested: 5 ml per kidney of 5 and 1 μM (for both the syngeneic and allogeneic transplants).

An important observation during the transplant surgery in this group was heavy bleeding at the site of the anastomosis, related to the use of PTL006. This was not noted in recipients of Soltran-treated kidneys. The bleeding was unexpected as the unique aspect of this therapy is that the modified inhibitor has a novel synthetic tail which tethers the therapeutic agent (here an anti-thrombin) to the phospholipid bilayer of cell membrane. This cytotopic delivery should minimize any systemic effects of administering the inhibitor that could harm the recipient, in this case prolonged bleeding. However, it appeared that some of the reagent was unbound to the donor organ and upon blood reperfusion entered the circulation. Thus heavy bleeding occurred when the renal artery (not the vein) was anastomosed to the recipient circulation. Bleeding was also occurring at the ureteric anastomosis. Nonetheless, in these syngeneic and allogeneic transplants involving recipients, the heavy bleeding related to PTL006 was manageable and successful transplantation was achieved. Bleeding was controlled by prolonged and repetitive application of pressure and continuous swabbing with surgicel. When transplantation was performed in hyperimmune recipients, the same dose of PTL006 administered to the donor kidney was associated with uncontrollable bleeding at the arterial anastomosis, causing the death of the animal (under anaesthesia) or total ischaemia of the donor organ. This problem was exacerbated by an inherent difference in size of the two rat strains, Lewis rats being

significantly larger than age-matched DA rats. This resulted in controllable bleeding in unsensitised recipients. The vessel size disparity is exaggerated after the two month period required for Lewis sensitisation. In many cases, the renal artery of the sensitised Lewis rat was twice the diameter of the donor DA renal artery. Perfusion of the kidneys with lower doses of PTL006 (5 ml at 0.01 μM) reduced the bleeding, but efficacy was lost.

Further modification of the PTL006 infusion protocol by introducing two additional steps proved helpful. Rather than being immediately transplanted into the pre-sensitised recipient, the PTL006-treated kidney was allowed to rest on ice and kept moist with cold saline for a period of ten minutes. Following this, an additional perfusion (wash through) with 5 ml of Soltran (over 5 minutes) was performed to remove any unbound material. The dose of PTL006 used for these transplants was 5 ml at 2 μM per kidney. The modifications of the protocol resulted in a clear prolongation of the survival of the treated grafts (in hyperimmune recipients) to MST day 5 post-transplantation.

Binding of PTL006 in perfused DA kidneys transplanted into syngeneic recipients was confirmed using the fluorescent conjugate PTL006-FAM, which enabled direct visualization using a fluorescence microscope. Each kidney was perfused with 5 ml PTL006-FAM at 2 μM, and strong staining was detected on the glomerular capillary surface at 0.5 hours post-transplantation. Although the image was bright, it would have been possible to increases the sensitivity by using an antibody against the hirulog peptide (HLL) component of PTL006. This additional layer could have potentially made the signal stronger and possibly revealed staining in less obvious areas of the kidney such as the peritubular capillaries.

Having generated preliminary data using PTL006 at a dose of 5 ml at 2 μM per kidney, showing an effect on transplant survival in the primed Lewis rats, a further experiment with a larger number of transplants under identical conditions was carried out for the purpose of statistical analysis. The data generated showed that a single anticoagulant treatment of the graft led to prolonged graft survival (MST 4.6 days, n=8) in comparison to Soltran-treated grafts (MST 1.8 days, n=6). The pathological mode of rejection after PTL006 treatment of the organ appeared to switch from a pure, aggressive form of AMR (at 2 days post-transplantation) to a mixed picture with features of cell mediated rejection as well as AMR (at 5 days post-transplantation).

These features included lymphocytic infiltration of the renal tubules and arteries indicative of cell mediated rejection, and thrombotic microangiopathy consistent with AMR. As expected, Soltran-perfused kidneys rejected at day 2 post-transplantation exhibited marked histological features AMR with extensive thrombosis and overall infarction.

The mechanism by which PTL006 delayed and reduced the vascular component of injury can be related to the inhibition of thrombin by the active component (hirulog). Thrombin is a multifunctional serine protease that plays a pivotal role in coagulation. It recruits platelets and converts soluble circulating fibrinogen into fibrin monomers, which polymerize to form an insoluble blood clot. Thrombin is short lived in the circulation; however under pathogenic conditions accumulation of coagulation factors with extravascular Tissue Factor initiates the enzymatic process of coagulation. As a result, thrombin acts near the disrupted site at which it is produced (Esmon CT et al., 1999 **310**). In this model, thrombin is generated when alloantibody is deposited onto the transplanted endothelium. Upon treatment of the graft with PTL006, the procoagulant effects mediated by thrombin are likely to have been inhibited by reducing formation of a platelet plug and fibrin clot. Since PTL006 was shown to be retained by the small vessels of the graft at 0.5 hours post-reperfusion, its therapeutic effects will have been active during the window in which HAR occurs. In addition, since thrombin has the ability to amplify its own activity, the use of PTL006 could prevent feedback activation of coagulation, which is likely to have contributed to the severity of vascular injury seen in the untreated model.

The therapeutic effects of PTL006 on graft survival and minimizing injury could also have been due to inhibition of the pro-inflammatory properties of thrombin. A study reported by Chen D et al. sheds some light on this. In a xenotransplantation setting, mice hearts were transplanted into rats that had been treated with ANCROD, thus depleting fibrinogen. However, fibrinogen-depletion had no effect on the prolongation of survival or influx of inflammatory cells (predominantly macrophages and NK cells). In the same study, parallel experiments were carried out involving transplantation of hearts from transgenic mice expressing endothelial tethered anti-coagulant hirudin protein, into rat recipients. The results showing prolonged graft survival and marked reduction of inflammatory cells and expression of MCP-1 highlighted the anti-

inflammatory properties of thrombin blockade, as opposed to the impact of fibrinogen depletion (Chen D et al., 2008 **311**). In a similar manner, the effects of PTL006 on the survival of the treated kidneys in primed Lewis rats may also have been due to targeting the proinflammatory properties of thrombin.

Thrombin signalling is mediated by a small family of G-proteins coupled to protease activated receptors (PARs) (Coughlin SR, 2000 **129**) and thrombin-mediated PAR receptors are expressed on the endothelium which, upon ligation, promote the release of chemokines (such as MCP-1). Indeed, thrombin, acting through PAR receptors, can have a variety of effects on endothelial cells, all contributing to cell injury. Thrombin causes the release of vWf from the endothelium which stimulates other proinflammatory responses, such as exocytosis of P-selectin (Lowenstein CJ et al., 2005 **100**). P-selectin is extremely important because it can mediate platelet aggregation and leukocyte adhesion (Yamakuchi M et al., 2007 **307**). In an *in vivo* model of cardiac allotransplantation, blocking P-selectin expression reduced monocyte recruitment mediated by the ligation of MHC Class I antibody to the endothelium (Valenzuela NM et al., 2013 **312**).

Thrombin is one of the most potent activators of platelets. Through interactions with the glycoprotein GP (Ib-V-IX) complex and vWf, the initial capture of platelets by the endothelium takes place. However, for sustained platelet activation, this process must be reinforced by locally generated thrombin. Inhibiting thrombin with PTL006 could potentially lead to platelet impairment. Platelets are pivotal to the rejection of vascularized organs (Kirk AD et al., 2009 **104**). Under the influence of potent agonists, especially thrombin, platelets release microparticles (MP) containing P-selectin contributing further to the inflammatory responses. Platelets not only express P-selectin but also CD40L (CD154) which further enhances their interaction with leukocytes. Thus treating DA kidneys with the coagulation inhibitor PTL006 prior to transplantation into pre-sensitised Lewis recipients, could have had a significant anti-platelet effect, dampening local inflammation that contributed to HAR in my model.

In AMR, it is known that complement activation is identified through C4d positive staining on the PTC. Complement inhibition alone did not have an effect on retarding the process of rejection. Complement may be activated in this model by the classical pathway, but in addition proteases of the coagulation cascade, especially thrombin, are

known to cleave pivotal complement components (e.g. C3 to C3a and C3b, or C5 to C5a and C5b) therefore amplifying complement activation (Markiewski MM et al., 2007 **186**). If, *in vivo*, thrombin can act as a convertase generating biologically active anaphylatoxins by direct cleavage of C3, C4 and C5, it will bypass standard complement inhibition at the convertase level. This would explain a lack of effect by complement inhibitor APT070 (Mirococept). As a result, inhibiting thrombin could potentially affect both complement-independent and complement-dependent mechanisms involved in HAR. Consequently, an extra group of DA transplants were prepared, in which the donor organ was treated with a combination of APT070 and PTL006 by intrarenal perfusion before transplantation into primed Lewis recipients. A non-significant additive effect was noted compared to the PTL006 alone treatment group, although according to the data generated previously, survival could only be attributed to the anticoagulant (PTL006) action. Finally, histological analysis of the kidneys treated with the combination therapy gave a very similar picture to that of PTL006-treated kidneys. A mixed immune response with elements of both cellular and humoral arms of rejection was observed. Indeed, the extent of cellular infiltration of the tubules and interstitium suggested that the T cell response against the donor organ made a significant contribution to the cause of graft rejection, whereas the untreated grafts were rejected though a humoral, antibody-mediated process.

The PTL006 cytotopic therapy introduced in this study does not directly target antibodies, nor does it have a known effect on lymphocytes, which in this model, start making an appearance at day 3 post-transplantation (data not shown). For this reason, the next chapter will focus on targeting the cellular responses (using standard immunosuppression) in animals that receive PTL004 (Thrombalexin)-treated kidneys. As noted elsewhere, PTL004 is the result of further development (as will be discussed later) of PTL006 with a number of potential advantages (including ease of synthesis).

Chapter 6 - Therapeutic Intervention using a novel Inhibitor of Coagulation PTL004 (Thrombalexin) in a Rat Model of HAR

6.1 Introduction

A newly developed anti-coagulant reagent called PTL004 (Thrombalexin) was produced using the same peptide sequence (hirulog-like peptide, HLL) as PTL006, but with a different membrane-inserting tail. As was mentioned in chapter 5, PTL006 has a polyethylene glycol (PEG) tail, which facilitates binding and insertion to the phospholipid bilayer and also increases agent solubility. In contrast, in PTL004, the HLL peptide is linked by a disulphide bond to a double myristoyl (bis) tail. The bis-myristoyl tail is likely to be better retained at the site of attachment than its counterpart (with a single myristoyl). Finally, PTL004 is quite a small molecule (4.6 kDa) – about the same size as insulin (5.8 kDa).

Following the same rationale, that by treating DA kidneys with this cytotopic reagent before transplanting into hyperimmune Lewis recipients, the immune insult to the endothelium mediated by thrombin's pro-coagulant and pro-inflammatory properties could be reduced, the effects of PTL004 were tested in this HAR rat model. Use of this potentially more efficient anti-thrombin reagent may further prolong graft survival. In addition, studies were extended to also target lymphocyte-mediated rejection, which in this highly sensitised model is observed from day 3 post-transplantation (data not shown). Specifically, the effects of standard immunosuppression (the calcineurin inhibitor Cyclosporine A or the mTOR inhibitor Rapamycin) with PTL004, were investigated.

6.2 Experimental Design

PTL004 was used in the pre-sensitised model of allotransplantation between DA donors and Lewis recipients. The experiments included DA kidneys perfused intrarenally with 2 μM PTL004 in 5 ml of, Soltran, before being transplanted into pre-sensitised Lewis recipients. The modified perfusion protocol was used for the intragraft delivery, which

included a 10-minute dwell-time and a final flush of the kidney to avoid excess bleeding at the site of arterial anastomosis. A control group was perfused with Soltran solution alone. Soluble anticoagulant (untailed HLL) was not used as a control to avoid the risk of systemic anticoagulation in the recipients. An additional group received 5 ml of 2 μM PTL004 and T-cell immunosuppression by Cyclosporine A (CsA). Administration of CsA to the recipient commenced 2 days prior to renal transplantation at a dose of 10mg/kg/day given intraperitoneally and continued daily until rejection of the transplant, as defined in Chapter 2. A control group of pre-sensitised Lewis rats received the CsA but kidneys were perfused with Soltran alone (no PTL004). Alternatively, Rapamycin was also used as the T-cell immunosuppressive reagent in a second set of experiments. Presensitised Lewis rats received PTL004-treated DA kidneys with simultaneous intraperitoneal administration of Rapamycin at a dose of 1.5mg/kg/day, on the day of transplantation. Rapamycin therapy continued daily, until the end of the experiment. A control group comprised of pre-sensitised Lewis rats that received Rapamycin and Soltran-treated kidneys. Following transplantation, survival of the transplant was monitored as before, including renal function measured by daily serum analysis of blood urea nitrogen (BUN). Graft pathology was also assessed in the experimental groups described.

Finally, for mechanistic evaluation, a third group of DA kidneys were perfused with PTL004 and transplanted into hyperimmune Lewis recipients, and in this case the recipients were then sacrificed at 24 (n=2) and 48 hours (n=2) post-transplantation so that the transplant organ could be stained for MAC (C5b-9) before the organ had ben rejected. In this experiment, the aim was to determine whether inhibition of coagulation (thrombin) had an effect on complement activation.

6.3 Graft Survival in Pre-sensitised Lewis Recipients of PTL004-treated Kidneys from DA donors

DA donor kidneys pre-treated with 5 ml of 2 μM PTL004 had a significant prolongation of survival in hyperimmune Lewis recipients (MST 5.1 days), compared to the recipients that received Soltran-perfused kidneys (MST 1.5 days).

The PTL004-treated group (n=6) exhibited 100% graft survival at 24 hours post transplantation, as opposed to 50% for the Soltran-treated group (2 of 4). The remaining donor organs of the control-treated group survived to 48 hours post transplantation (2 of 4). In contrast, of the animals that received PTL004-treated kidneys (3 of 6), 50% had extended graft survival to day 5 post transplantation. The survival of the remaining animals was equally distributed (1 of 6) between days 3, 6 and 7 (16.6% each) (Figure 6.1).

PTL004, in a perfusion-concentration of 2 μM, was therefore effective in prolonging graft survival in hyperimmune recipients. By comparison with earlier results for PTL006 (Chapter 5), there was no clear difference in graft survival. This indicated that regardless of the tail possessed by the anticoagulants (PEGylated in PTL006 and bis-myristoyl in PTL004) cell tethering was equally effective.

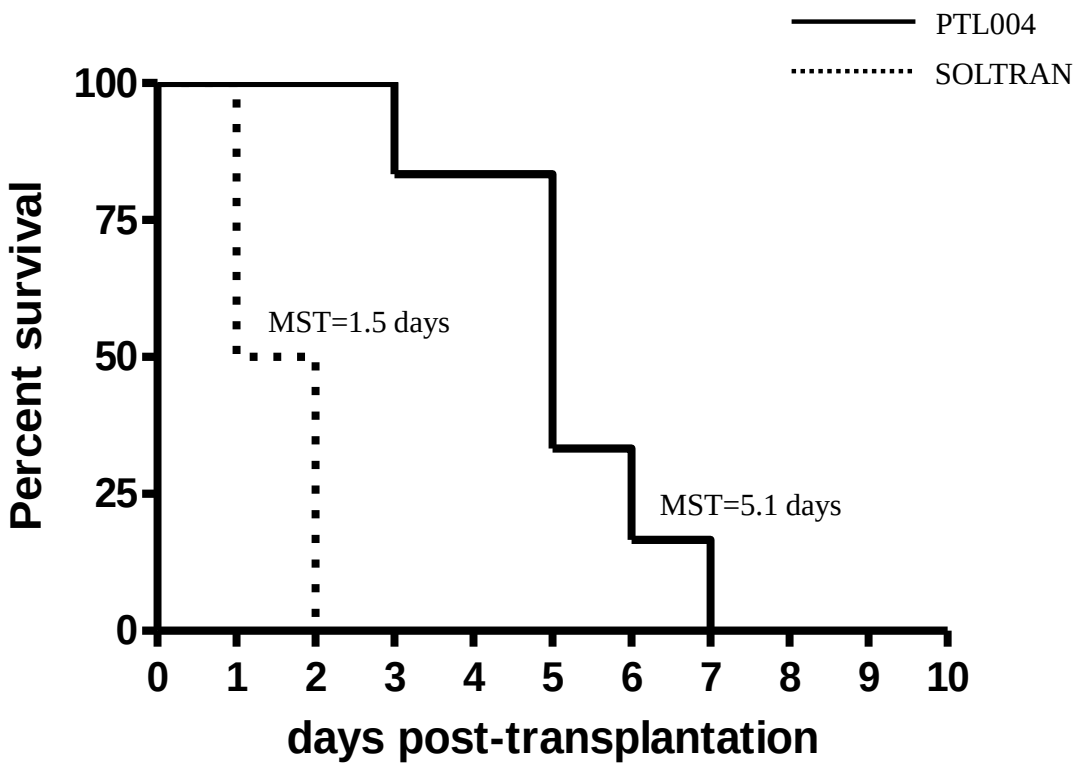


Figure 6.1: Graft survival following transplantation of DA kidneys treated with PTL004 into pre-sensitised Lewis recipients. PTL004 treatment of donor organs at a dose of 5 ml at 2 μM (n=6) achieved significant prolongation of survival compared to Soltran-treated control group (n=4) (p=0.0018).

6.4 Renal Function in Pre-sensitised Lewis Recipients of PTL004-treated Kidneys

Renal function in the Lewis recipients was monitored daily post transplantation until graft loss. BUN measurement showed the decline in renal function after transplantation was delayed, relative to the decline in recipients of Soltran-treated control transplants (Figure 6.2 A). Significant protection by PTL004 therapy was evident at 24 hours post transplantation. The average BUN value in the Soltran-treated group was >35 mmol/L whereas the BUN for kidneys that received PTL004 was normal, i.e. <10mmol/L at this time (Figure 6.2 B).

Kidney protection with 5 ml of 2 μM PTL004 was therefore effective at postponing the onset of graft rejection associated with high–titre anti-MHC antibodies in this study.

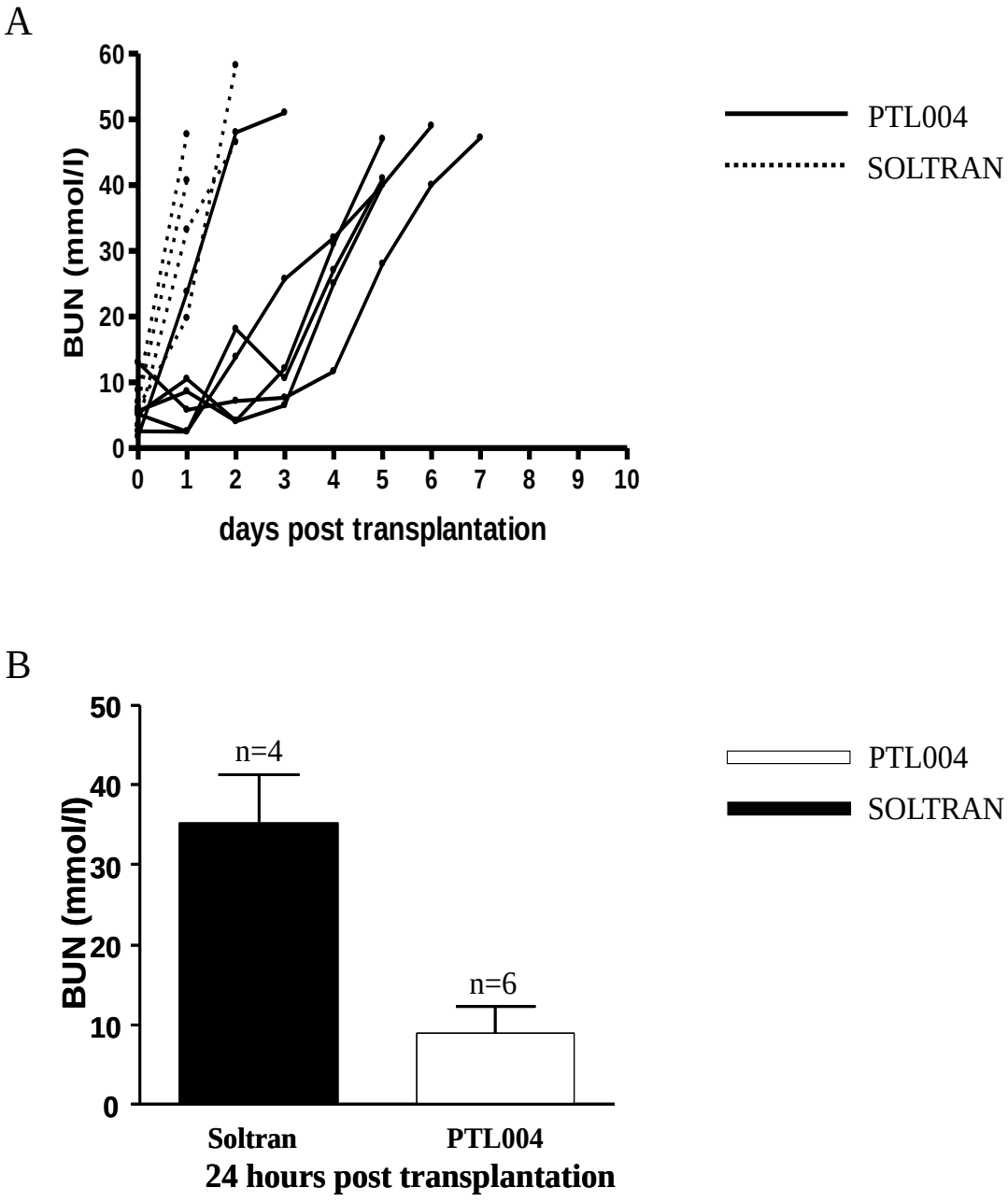


Figure 6.2: Renal function after PTL004 intervention. A: PTL004 treatment of DA donor organs, at a dose of 5 ml at 2 μM, prevented immediate deterioration of renal function following transplantation into hyperimmune Lewis recipients. Control treatment with Soltran was ineffective. B: There was significant improvement of BUN (lower normal levels) at 24 hours post transplantation (p=0.0190) for the PTL004-treated group.

6.5 Histology of PTL004-treated DA donor Kidneys rejected by Pre-sensitised Lewis Recipients

Since the MST for PTL004-treated grafts was 5.1 days and for control grafts was 1.5 days, histology of rejected PTL004-treated grafts was possible at day 5 (n=3) and on day 2 post-transplantation for the control group (n=2).

Results for the PTL004-treated group showed evidence of both cell-mediated rejection (lymphocytes in the tubules and glomeruli) and antibody-mediated rejection (inflammatory cells in the PTC and fibrin in the microvasculature) at day 5 post-transplantation (Figure 6.3). However, the overall impression was that there was less thrombosis although a quantitative study was not carried out. In contrast the Soltran-treated kidneys at day 2 post-transplantation showed only antibody-mediated rejection, no lymphocytic infiltrates (Figure 6.4).

This assessment of graft morphology revealed that a single intrarenal delivery of PTL004 was able to slow the rejection process from an aggressive, accelerated antibody-mediated rejection to a less vigorous acute antibody and cellular rejection. This could be significant in the clinic.

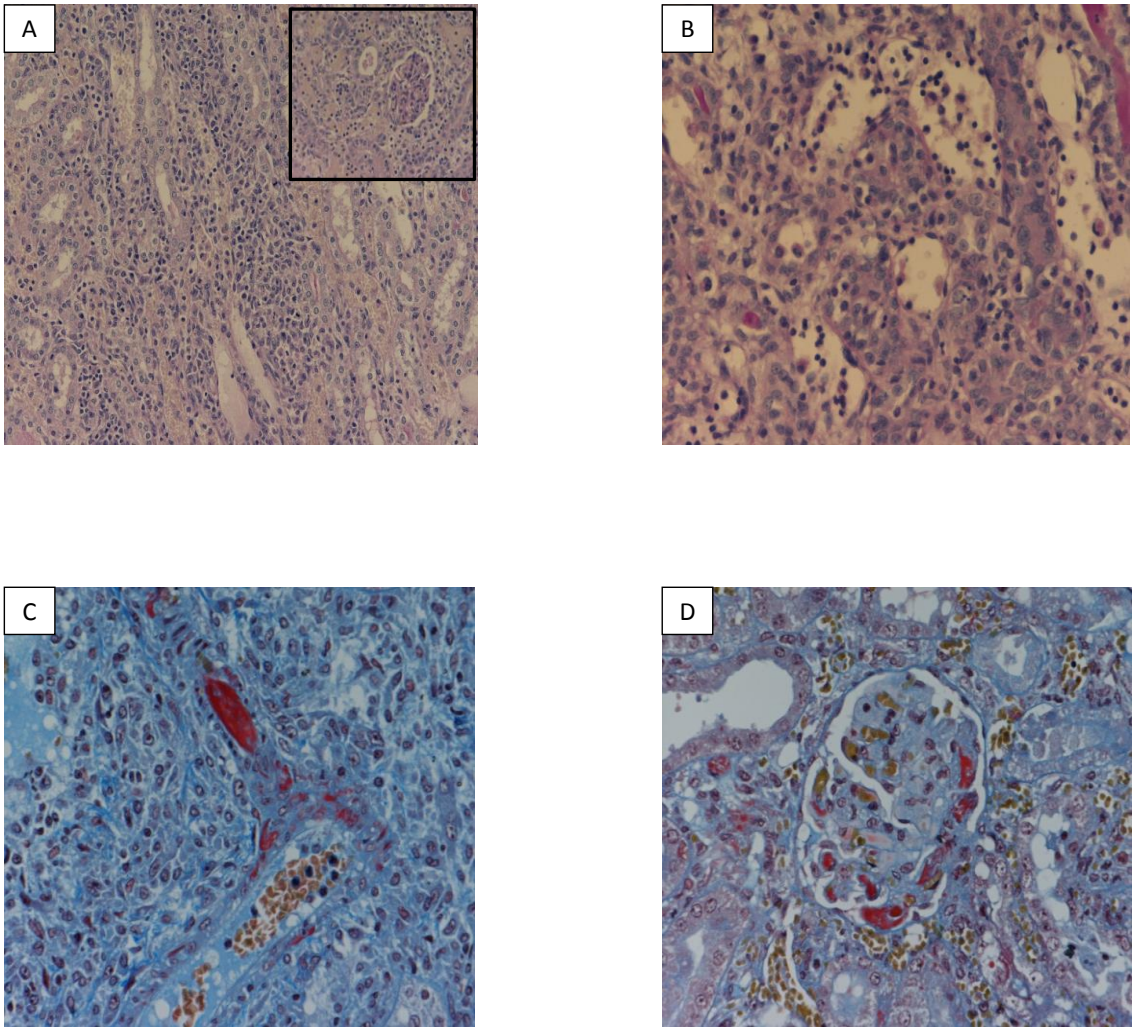


Figure 6.3: DA donor kidneys treated with PTL004 and rejected by pre-sensitised Lewis recipients on day 5 post-transplantation (n=3). A: Typical section showing signs of lymphocyte-mediated rejection in the form of tubulitis (PAS; x200) and glomerulitis (inset) (PAS; x400). B: Extensive peritubular capillaritis with presence of monocytes in the PTC (PAS; x400). C: Small vessel fibrinoid necrosis (MSB; x400). D: Glomerular thrombosis (MSB; x400).

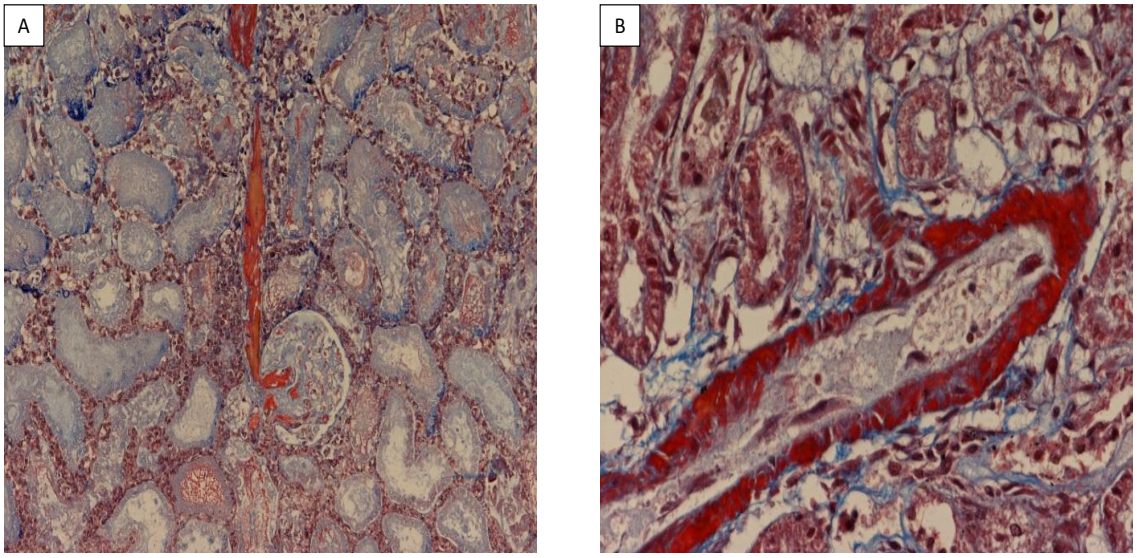


Figure 6.4: DA donor kidneys treated with Soltran and rejected by pre-sensitised Lewis recipients at day 2 post-transplantation (n=2). A: Representative allograft showing features of HAR with infarcted area of tubules, thrombosed glomerulus and arteriole (MSB; x200). B: Extensive fibrinoid necrosis on arterial wall, neutrophilic and monocytic infiltration on the PTC and general infarction (MSB; x400).

6.6 Impact of CsA or Rapamycin on survival of PTL004-treated Grafts in Hyperimmune Recipients

In section 6.3 it was shown that most PT004-treated grafts were rejected by hyperimmune rats between days 5-7 post transplantation, coinciding with a dense lymphocytic infiltrate. My hypothesis for the next set of experiments was that control of T cell mediated rejection using conventional T cell suppressive agents would extend the graft survival beyond that enabled by PTL004 graft treatment alone. Cyclosporine A (CsA) was initially used, as this calcineurin inhibitor has an established record of suppressing T cell mediated rejection, in the clinic and in experimental animals.

CsA treatment of pre-sensitised Lewis recipients commenced 2 days before transplantation, using a dose of 10mg/kg/day intraperitoneally. The treated hyperimmune rats then received a DA kidney pre-treated with 5 ml of 2 μM PTL004 in Soltran (n=8). Daily CsA treatment continued until graft loss. Graft loss in animals that received this combination therapy was unexpectedly biphasic. 50% of the animals (n=4) rejected the kidneys in an accelerated fashion whereas 50% had prolonged survival in comparison to CsA treatment alone, i.e. the kidney was perfused only with Sotran (n=6) (Figure 6.5). In detail, 37.5% of recipients that received both CsA and PTL004, lost their graft at 24 hours post-transplantation (3 of 8) and one (12.5%) had graft loss by 48 hours post-transplantation (1 of 8). In contrast, the remaining 4 animals of the PTL004/CsA group had substantially extended survival. 12.5% of the animals survived to day 7 post-transplantation (1 of 8) whereas 25% survived to day 8 post-transplantation (2 of 8). Remarkably for this model, 12.5% (1 of 8) survived to day 10 post-transplantation. Overall, the MST of the PTL004/CsA group was 4.8 days post transplantation and did not result in significant prolongation of graft survival compared to the control group. Animals that received CsA treatment and Soltran-perfused kidneys rejected their grafts in an accelerated manner, as expected. This meant 66.7% of the grafts had failed at 24 hours post-transplantation (4 of 6) and the remaining 33.3% grafts had failed at 48 hours post-transplantation (2 of 4), with an MST of 1.3 days post-transplantation. Thus, CsA in the stated dose had no overall benefit in this pre-sensitised model. However, this calculation of the mean survival time may be misleading, since the graft loss in the combined treatment group was biphasic, suggesting that in half of the group that benefit of PLT004 was actually lost.

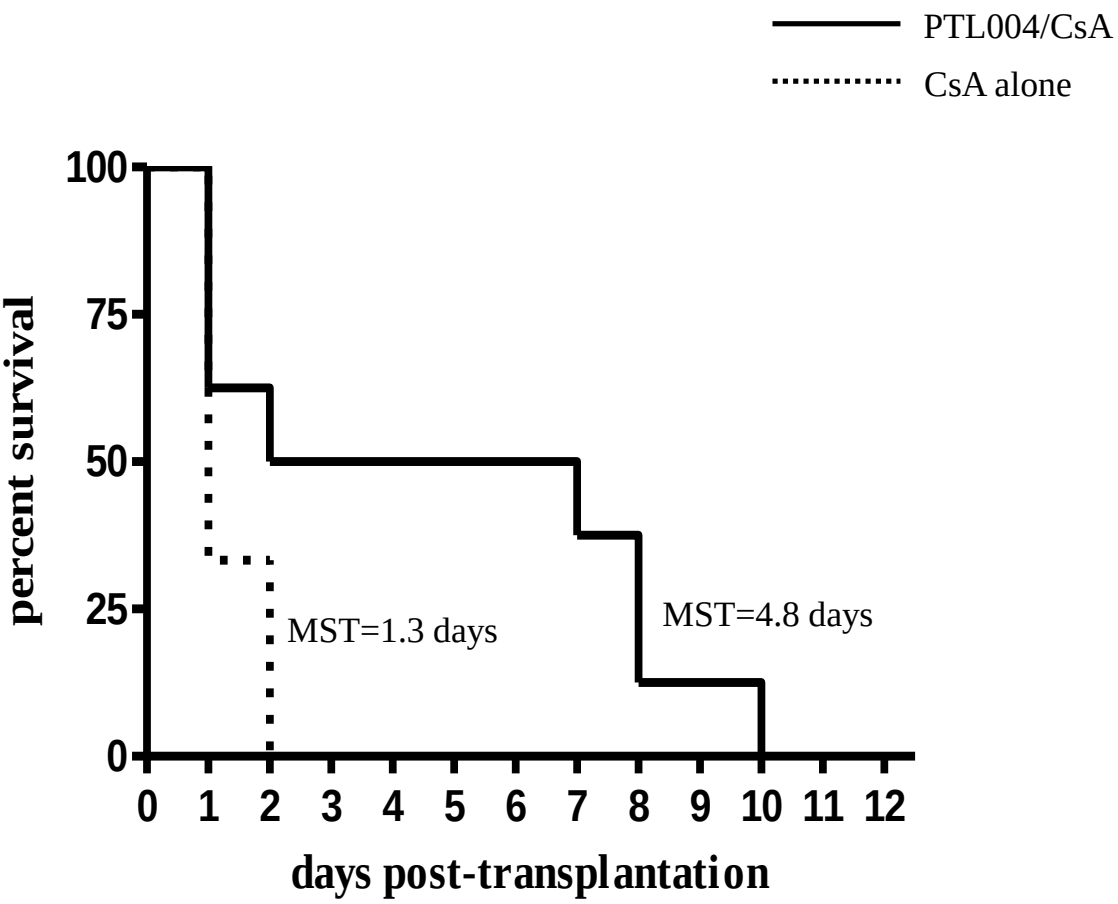


Figure 6.5: Graft survival curve for PTL004-treated DA donor organs transplanted into pre-sensitised CsA-treated Lewis recipients. The combination of PTL004 (5 ml of 2 μM) treatment of donor organ and CsA (10mg/kg/day) treatment of the recipient (n=8) failed to significantly prolong graft survival compared to treatment with CsA alone, i.e. where the donor organ was Soltran-treated (n=6) (p=0.0633).

Paradoxically, one of the major limitations of CsA in clinical and experimental use is nephrotoxicity. It can cause acute necrosis of the renal tubule and less commonly can mediate acute endothelial injury of the donor vasculature (Davies DR, 2000 **313**), Malyszko J et al., 1996 **314**). Although the dose of CsA used in my experiments was based on successful use in rat kidney transplant models (Chen D et al., 2008 **311**), it is possible that endothelium could have been sensitised by CsA to antibody mediated rejection and hence offset the beneficial effect of PTL004 identified in the absence of

CsA. Nonetheless, the inconsistent results and overall failure of CsA to extend graft survival prompted a change of immunosuppression to Rapamycin.

In the following exploratory experiment rapamycin was administered intraperitoneally at a dose of 1.5mg/kg/day to the pre-sensitised Lewis recipients on the day of transplantation. The DA donor kidney was perfused with 5 ml of 2 μM PTL004 before implantation. Rapamycin treatment continued daily until graft failure.

With this combined treatment of PTL004 and Rapamycin (n=6), graft survival was significantly prolonged compared to Rapamycin alone in recipients of a Soltran-treated kidney (Figure 6.6). Namely, in the combined treatment group, graft failure was equally distributed (16.7%) between days 1, 4, 5 and 9 (1 of 6 at each time point) whereas 33.3% of animals had extended graft survival to day 8 post-transplantation (2 of 6). MST was 5.8 days post-transplantation compared to the group that received Rapamycin and Soltran-perfused kidneys (n=4) with an MST of 1.5 days post-transplantation (Figure 6.6). In this group, 50% survived 24 hours (2 of 4) and 50% to 48 hours post-transplantation (2 of 4).

In conclusion, the combination of Rapamycin treatment of the recipient and PTL004 treatment of the donor kidney was associated with enhanced graft survival compared with Rapamycin treatment alone. The MST achieved with this combination was amongst the best out of the other treatment protocols (Figure 6.1) and the results with Rapamycin were more consistent than with CsA in this combined treatment model (Fig 6.5). The next step would have been to optimize the combined treatment protocol (of Rapamycin/PTL004) by direct comparison with PTL004 alone and also by escalating the dose of Rapamycin. However time did not permit these experiments in my thesis plan.

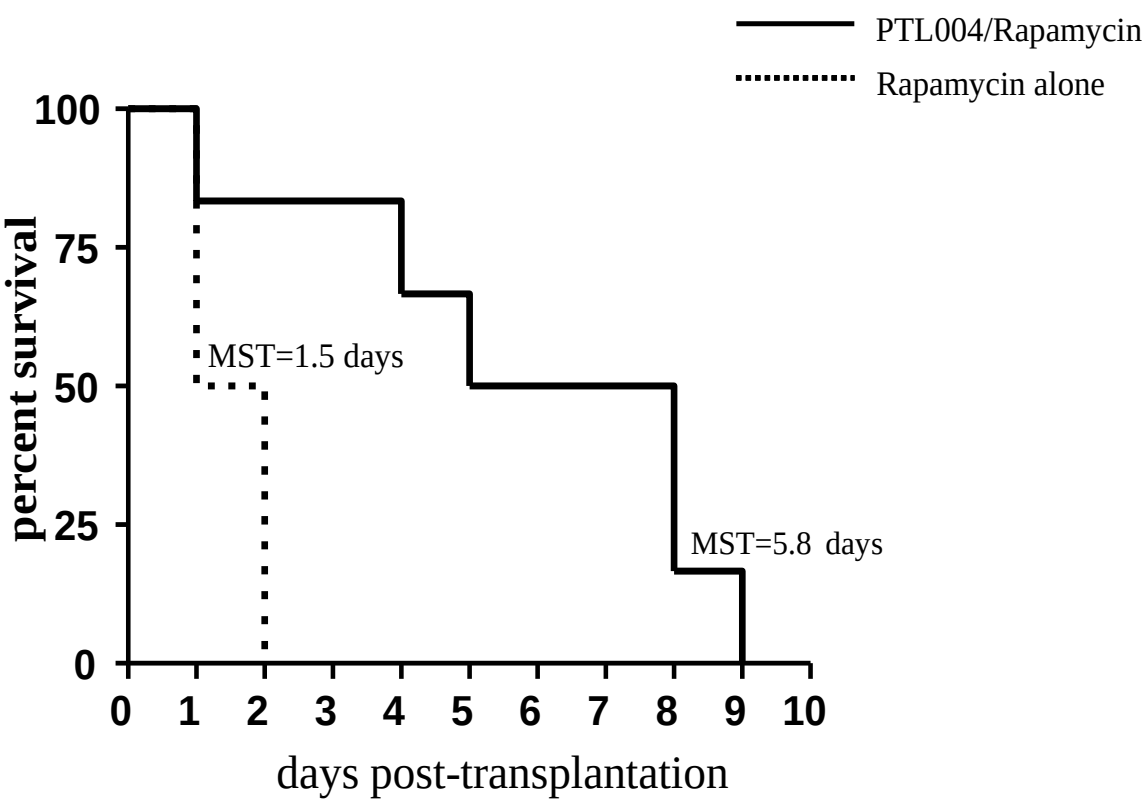


Figure 6.6: Graft survival following transplantation of DA kidneys treated with PTL004 into pre-sensitised Lewis recipients receiving Rapamycin. The combination of PTL004 (5 ml of 2 μM) treatment of donor organs and Rapamycin (1.5mg/kg/day) treatment of the recipients (n=6) achieved significant prolongation of graft survival (p=0.0188) compared to recipients that received Soltran perfused kidney and Rapamycin treatment (n=4).

6.7 Renal Function in Pre-sensitised Lewis Recipients treated with CsA or Rapamycin and transplanted with PTL004-treated DA Donor Kidneys

In both combination groups, PTL004/CsA and PTL004/Rapamycin, renal function in the Lewis recipients was monitored by BUN measurement on each day after transplantation until the time of transplant failure. The treatment protocols for CsA, Rapamycin and PTL004 are described in Section 6.2.

For the CsA group, BUN levels are shown in figure 6.7. BUN measurements identified two divergent sub-groups in the combined PTL004/CsA: those with rapid onset graft failure and those with delayed graft failure (Figure 6.7 A). Taking the group as a whole, however, at 24 hours post-transplantation BUN levels were significantly lower in the PTL004/CsA group compared to the CsA/Soltran treatment group (Figure 6.7 B).

For the Rapamycin group, BUN levels appear in figure 6.8. Combined treatment with PTL004/Rapamycin was associated with delayed onset and a slower progression of renal transplant failure compared to Rapamycin/Soltran treatment group (Figure 6.8 A). Significant protection against graft failure was afforded by PTL004/Rapamycin therapy at 24 hours post-transplantation in comparison to the Rapamycin-treated group receiving Soltran-perfused kidneys (Figure 6.8 B).

Consistent with the findings on graft survival, these functional measurements showed no overall benefit of CsA treatment in the recipient added to treatment of the donor organ with PTL004, when compared with PTL004 alone, although there was a transient benefit at 24 hours post-transplantation. In contrast, substitution of CsA with Rapamycin caused a more gradual onset of renal impairment compared to the Soltran/Rapamycin group; nonetheless, functional deterioration was observed in all animals by day 9 post-transplantation.

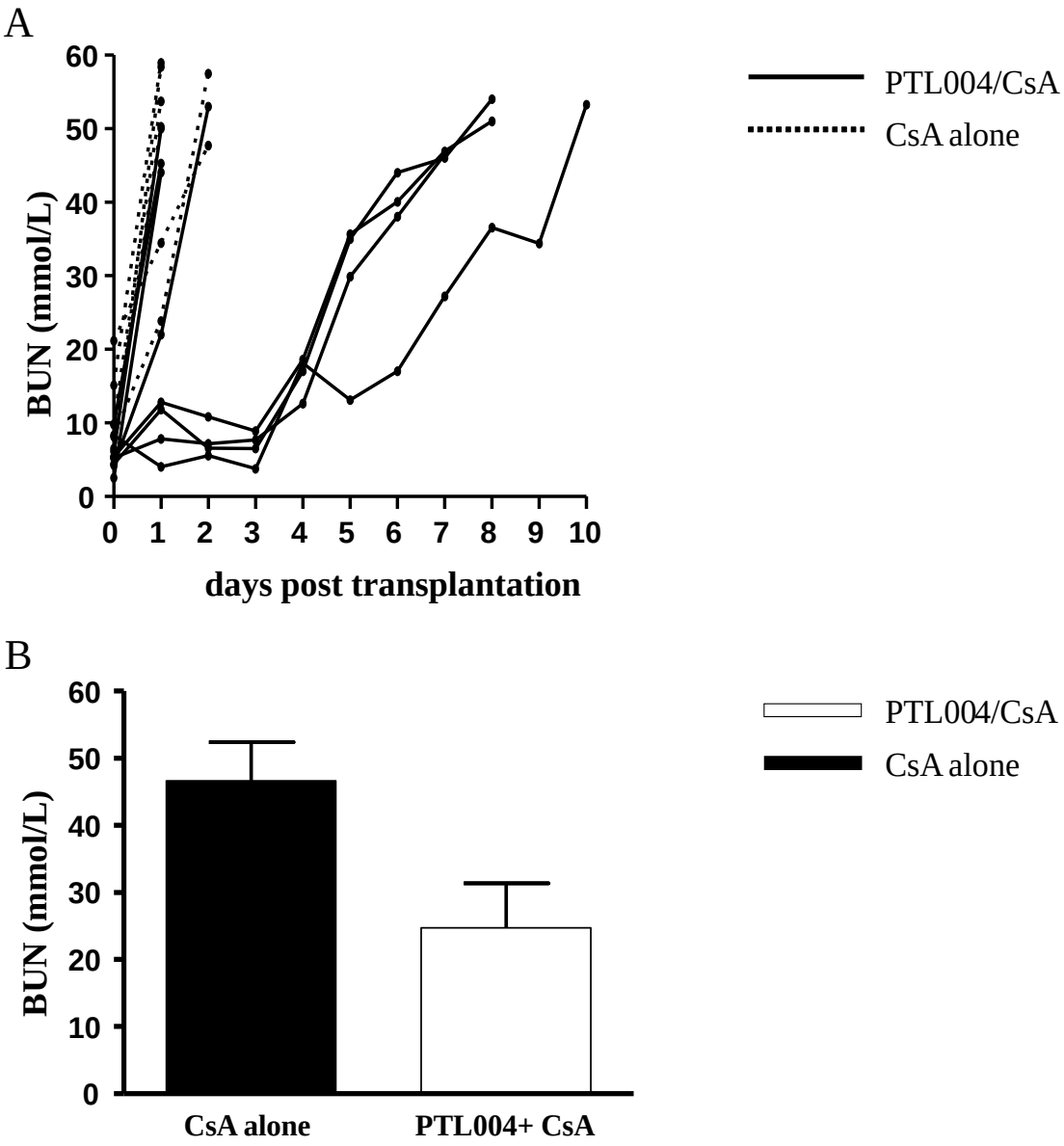


Figure 6.7: Renal allograft function with PTL004/CsA intervention. A: PTL004/CsA treatment led to improved renal function in 50% of the transplanted hyperimmune Lewis recipients compared with the Soltran/CsA-treated group. B: BUN measurement at 24 hours post-transplantation showed better renal function in the PTL004/CsA group compared to Soltran/CsA controls (p=0.02).

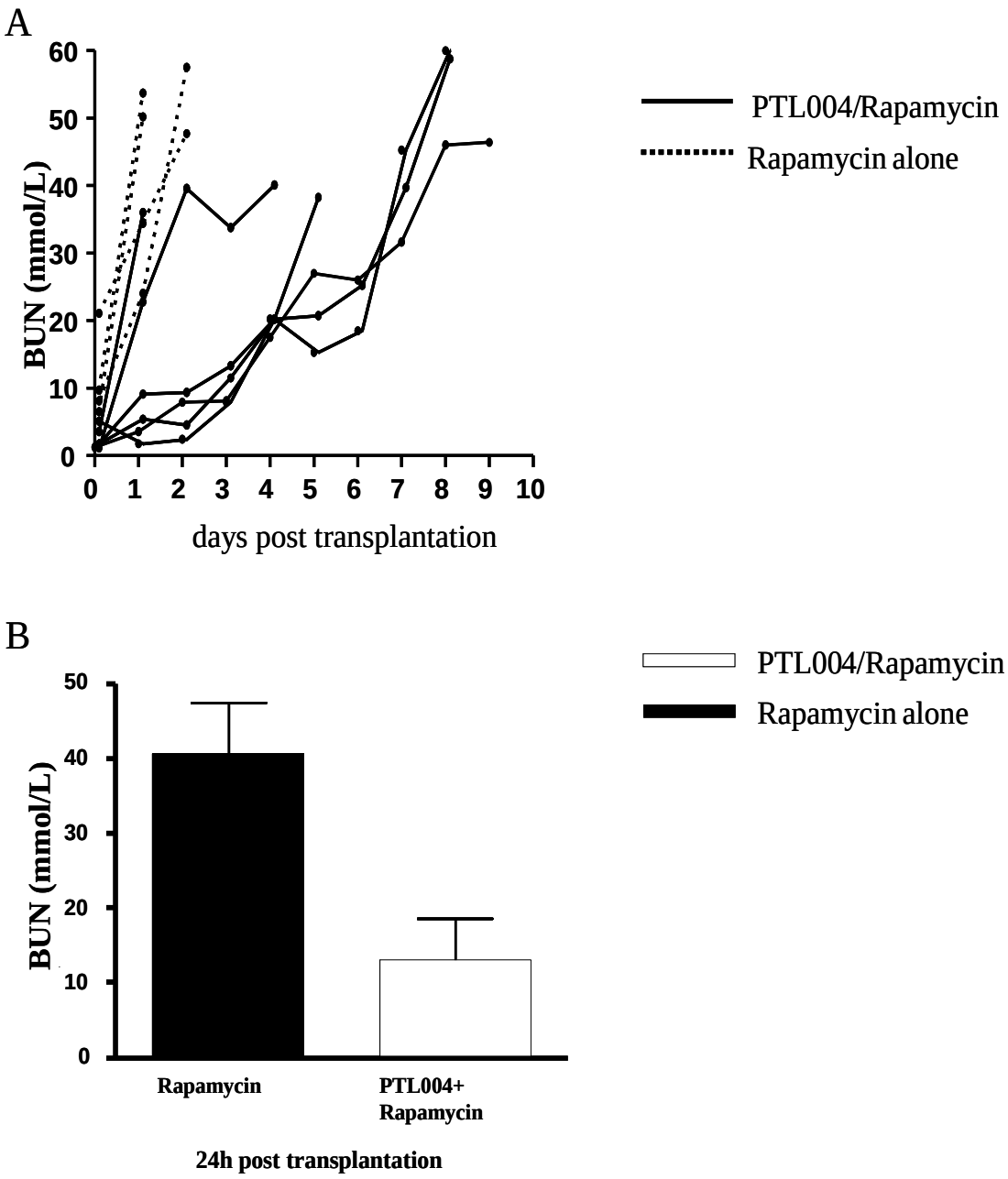


Figure 6.8: Renal allograft function with PTL004/Rapamycin intervention. A: PTL004/Rapamycin treatment improved renal function compared with the Soltran/Rapamycin-treated group. B: There was marked protection against transplant failure at 24 hours post-transplantation (p=0.0381) in the PTL004/Rapamycin treatment group compared with Soltran/Rapamycin controls.

6.8 Histology of PTL004-protected grafts transplanted into Hyperimmune Recipients treated with CsA or Rapamycin

In the CsA study, pathology was assessed in the graft tissue from animals that had received 5 ml of 2 μM-PTL004-perfused kidneys and daily intraperitoneal injections of CsA at 10mg/kg/day.

In the sub-group of 4 transplants with rapid rejection (50% of the whole group treated with CsA/PTL004), histological examination showed evidence of extreme infarction and vacuolated renal tubules, a phenotype often associated with CsA-induced nephrotoxicity. Vacuolation of renal tubules was extensive. In addition, the presence of haemorrhage and thrombosis in association with neutrophils in PTC was indicative of antibody-mediated rejection (Figure 6.9). Toxicity mediated by CsA administration could explain why transplant survival in this sub-group was poor.

In the CsA/PTL004 subgroup with prolonged graft survival (n=4), the renal transplant tissue was substantially better preserved, even though the features of CsA-toxicity were present. In these recipients, areas of renal vacuolation were widely distributed in the tissue. There were also some signs of AMR such as glomerulitis and peritubular capillaritis. There was a clear absence of lymphocytes, even from the late graft losses, indicating that CsA treatment had been effective in suppressing T cell-mediated rejection (Figure 6.10).

CsA alone in the absence of cytotopic therapy (with PTL004) offered no protection against HAR, as portrayed in the histology results (for rejected grafts, by day 2). These showed severe infarction and also showed widespread tubule vacuolation and calcification, which is typical for CsA toxicity (Figure 6.11).

These data indicate that acute CsA-induced toxicity was a prominent feature in the whole group of transplants at the time of rejection, even those rejected within the first 2 days of transplantation. CsA could possibly have played a role in the abnormal results generated in the group that received combined treatment of PTL004 and CsA indicating that for this model CsA might not have been a suitable choice as a T-cell immunosuppression reagent.

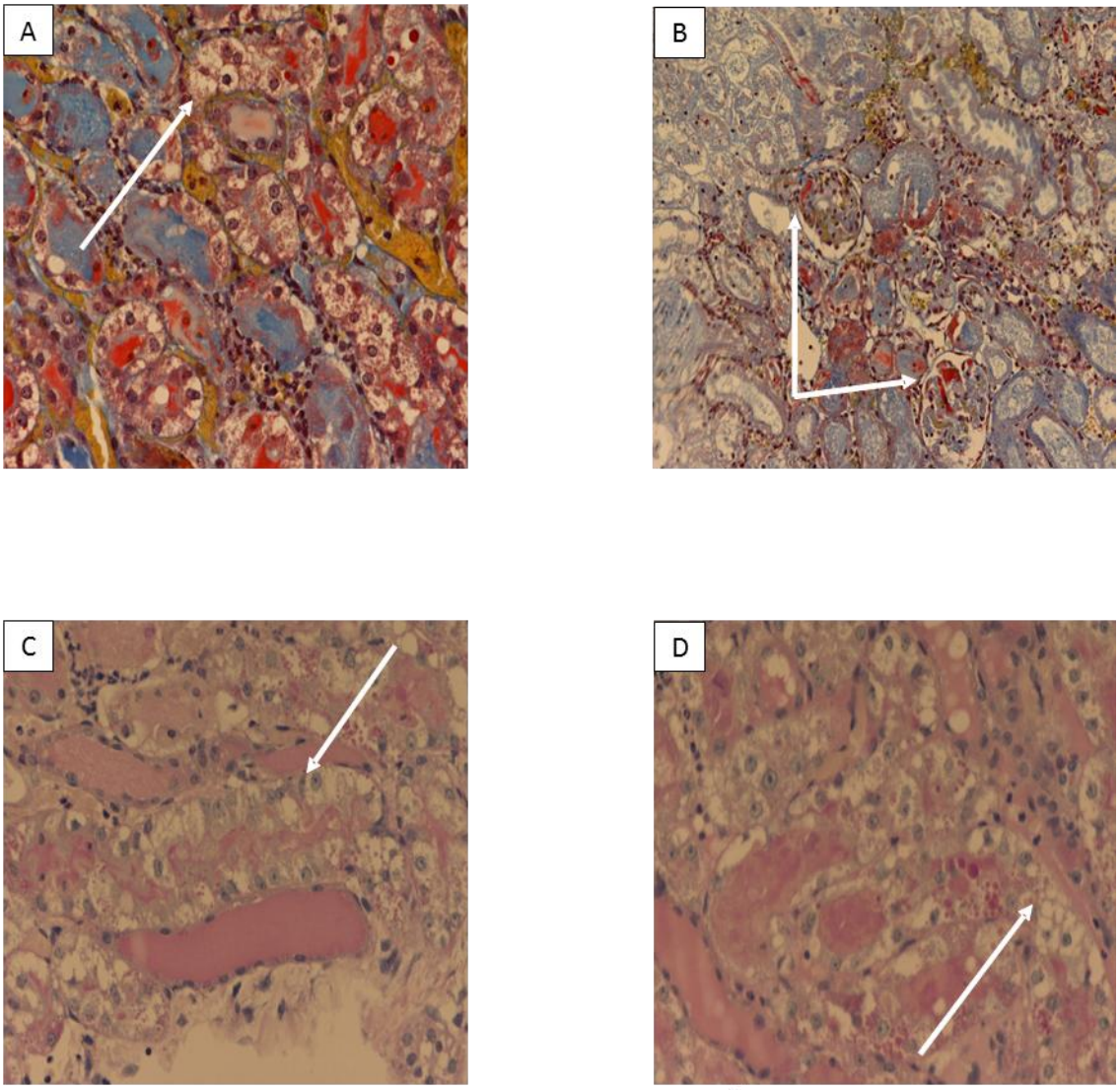


Figure 6.9: An example of donor kidneys treated with PTL004 and grafted into pre-sensitised recipients treated with CsA, rejected in an accelerated manner (n=4). A: Histology of a representative DA kidney rejected at day 1 post-transplantation despite treatment with PTL004/CsA and showing marked vacuolation of tubules, a possible indication of CsA toxicity (MSB; x400). B: Extensive infarction and thrombosis (MSB; x200). C: Graft rejected at day 2 post-transplantation with tubules showing infarction and vacuolation (PAS; x400). D: Vacuolation (PAS; x400).

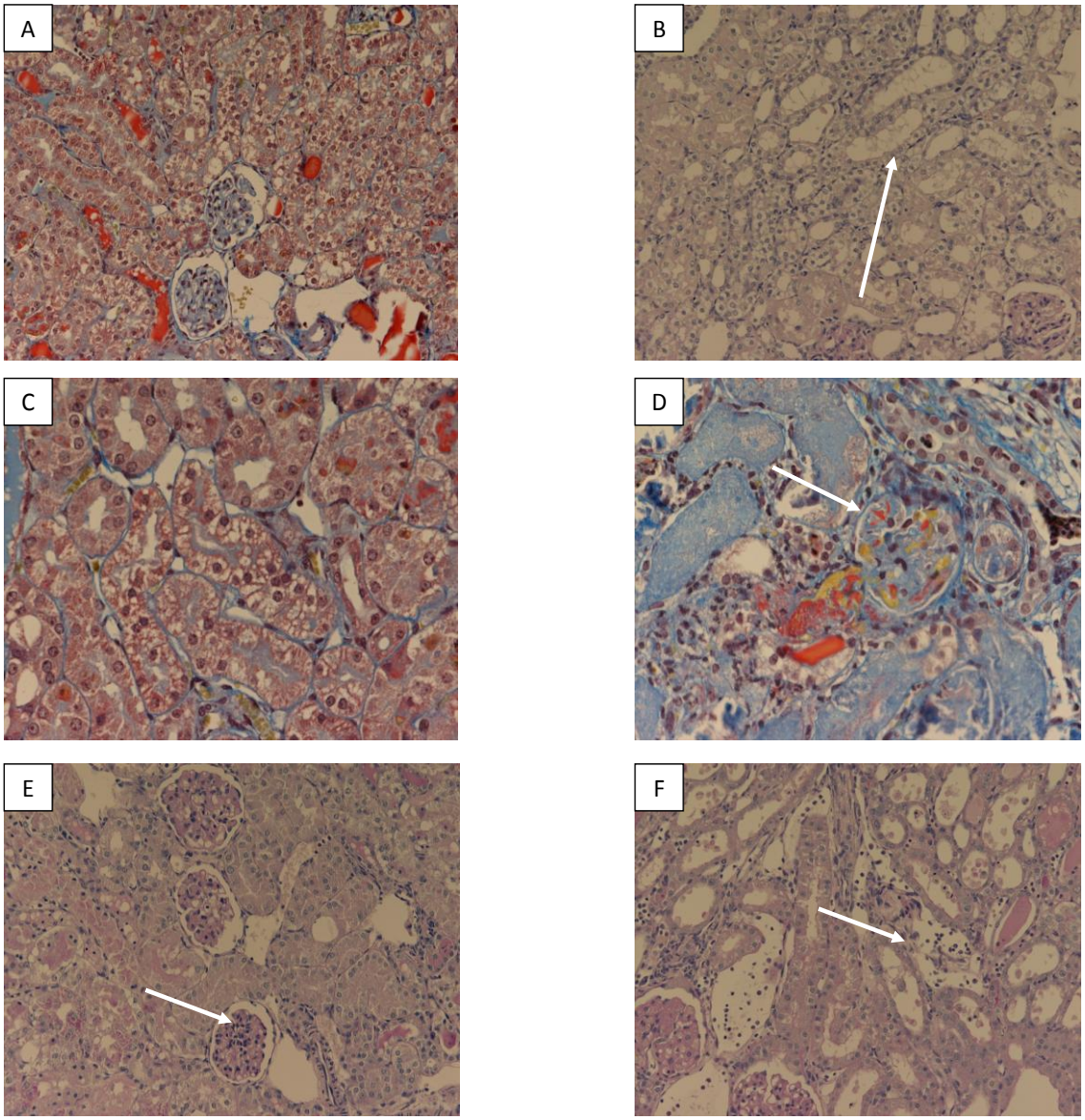


Figure 6.10: Examples of donor kidney treated with PTL004 and grafted into pre-sensitised recipients treated with CsA, with prolongation of survival (n=4). A: Representative DA allografts rejected at day 8 post-transplantation showing vacuolation of tubules, a possible sign of CsA toxicity (MSB; x200). B: Renal tubule vacuolation (PAS; x200). C: Vacuolation (MSB; x400). D: Glomerular fibrin thrombi (MSB; x400). E: Mild glomerulitis with vacuolation of tubules (PAS; x200). F: Peritubular capillaritis (PAS; x200).

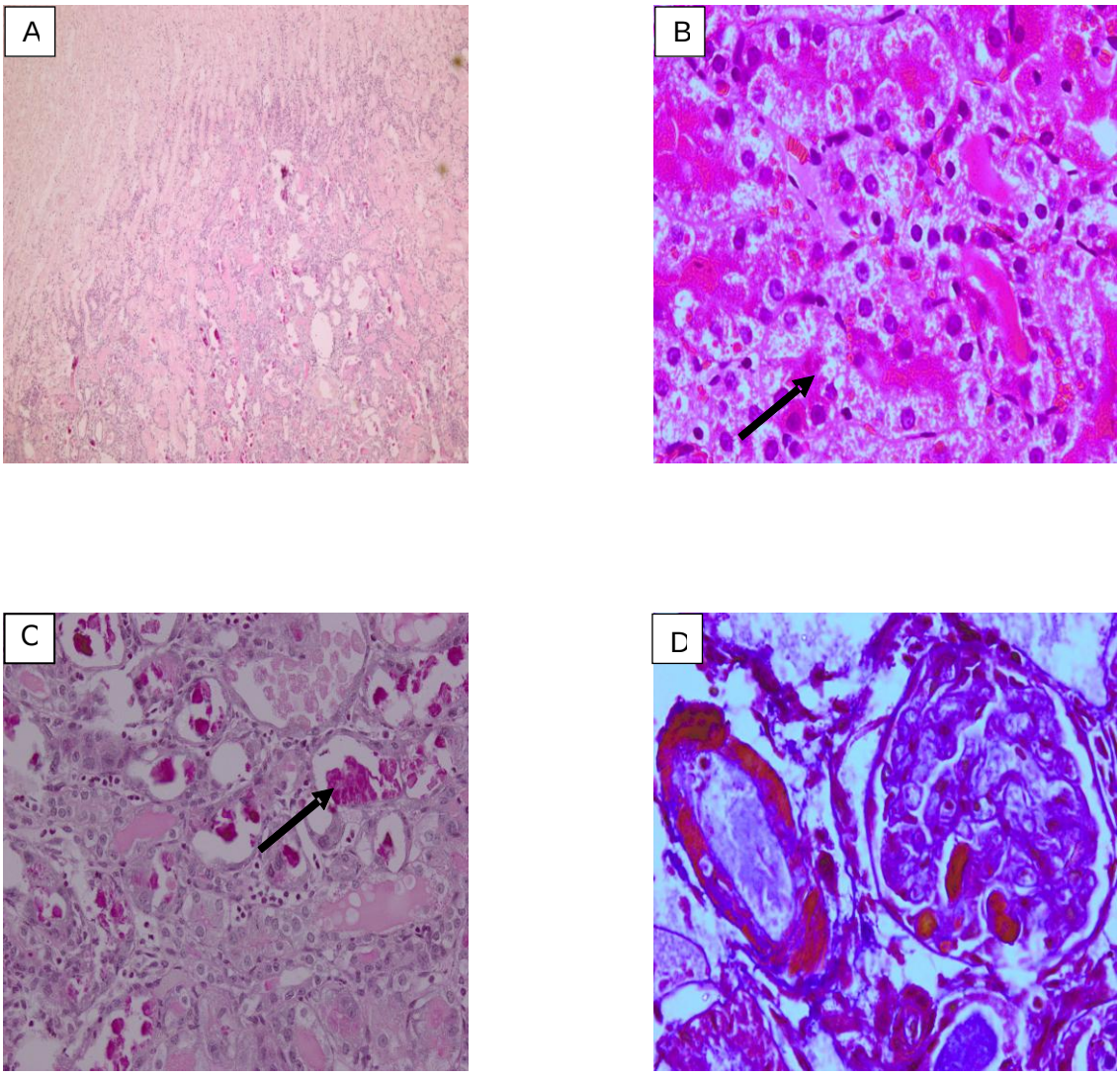


Figure 6.11 Soltran-perfused donor kidney transplants rejected by recipients treated with CsA (n=6). A: Histology of representative DA kidney allografts taken from a pre-sensitised Lewis recipient treated with Soltran/CsA at day 1 post-transplantation showing extreme infarction (PAS; x100). B: Renal tubule vacuolation (PAS; x400). C: Calcification of tubules (PAS; x200). D: Microvascular thrombosis (MSB; x400).

Due to possible harmful effects caused by CsA, the immunosuppressive regimen was changed to the mTOR inhibitor Rapamycin. The treatment regimens that included Rapamycin are described in Section 6.2. As described in Sections 6.6 and 6.7, Rapamycin, when given in combination with PTL004-treated kidney prolonged graft survival to give MST 5.8 days, compared to 1.5 days when Rapamycin was given to recipients of a Soltran-treated kidney.

For the combined Rapamycin/PTL004 treatment group, histological analysis is shown in figure 6.12. Here, representative micrographs are shown from animals that rejected their graft at day 5 (n=1), day 8 (n=2) and day 9 (n=1). Surprisingly, histological analysis for all time points revealed presence of extensive lymphocytic infiltration despite treatment with Rapamycin. Furthermore, all animals developed fibrinoid necrosis revealing the presence of ongoing AMR.

In contrast, for the Rapamycin-only treatment group (where the donor kidney was perfused with Soltran alone), rejected kidneys only showed features of HAR with thrombosis and necrosis (Figure 6.13)

In conclusion, treatment with the immunosuppressant Rapamycin did not lead to obvious cell toxicity visible by microscopy. Disappointingly, however, Rapamycin did not prevent lymphocytic infiltration of the graft. Rapamycin, therefore, may not effectively eliminate cellular immune responses in the dosing regimen and model used. It is not clear if there is any distinct advantage of adding Rapamycin over PTL004 alone, in terms of histological damage, since no direct comparison between Rapamycin/PTL004 and PTL004 alone was made.

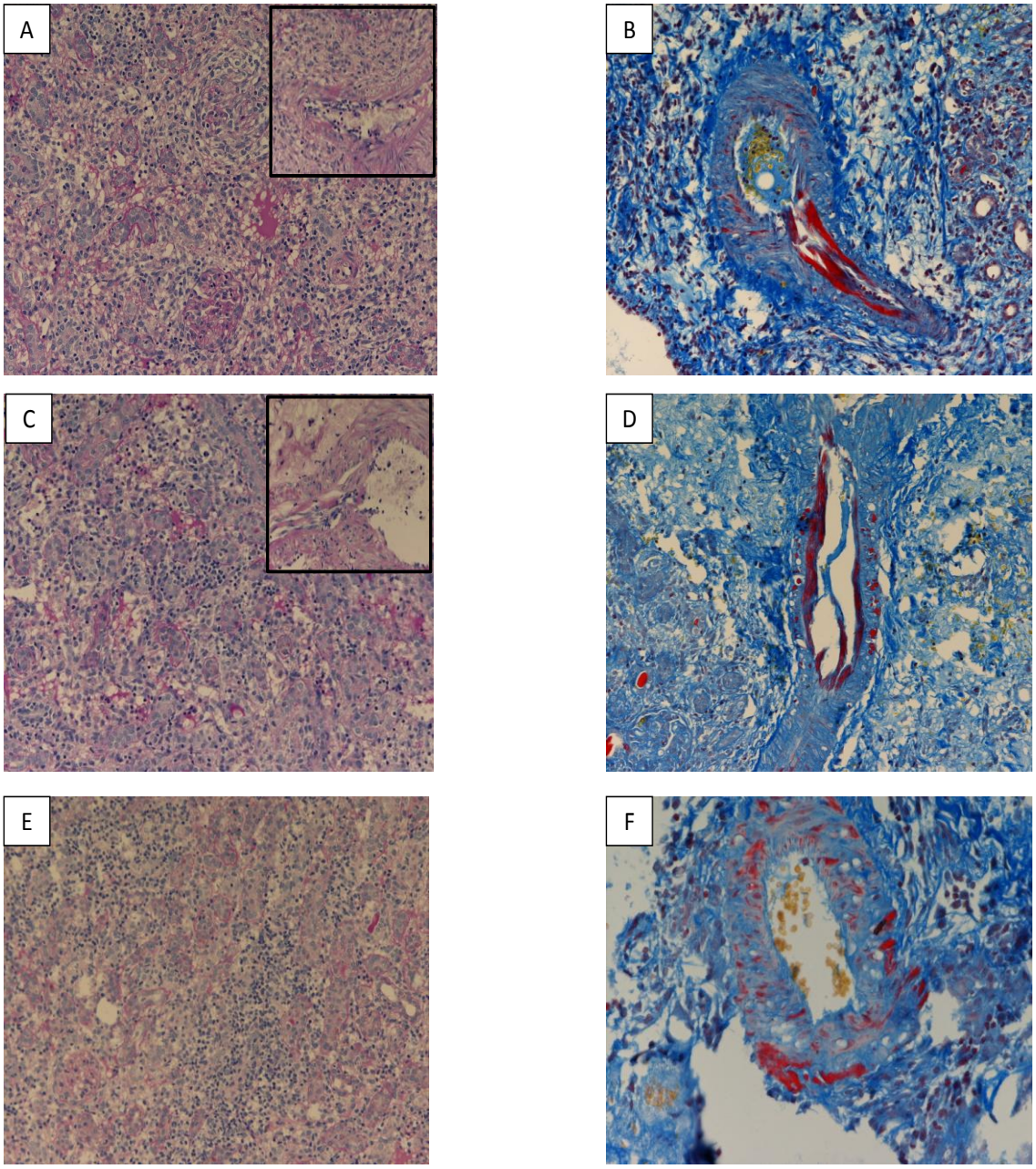


Figure 6.12: Sections from DA donor kidney allograft rejected by pre-sensitised recipients despite treatment with PTL004/Rapamycin. A: Allograft rejected at day 9 post-transplantation showing extensive lymphocytic infiltration, (PAS; x200). The inset shows arteritis (PAS; x400). B: Vessel wall from the same graft showing fibrinoid necrosis (MSB; x400). C: Allograft rejected at day 8 post-transplantation showing cellular rejection (PAS; x200). The inset shows arteritis (PAS; x400). D: Fibrin thrombi on the vessel wall of the same graft (MSB; x400). E: Allograft rejected at day 5 post-

transplantation showing widespread lymphocytic infiltration of the graft (PAS; x200). F: Fibrinoid necrosis in the same graft (MSB; x400).

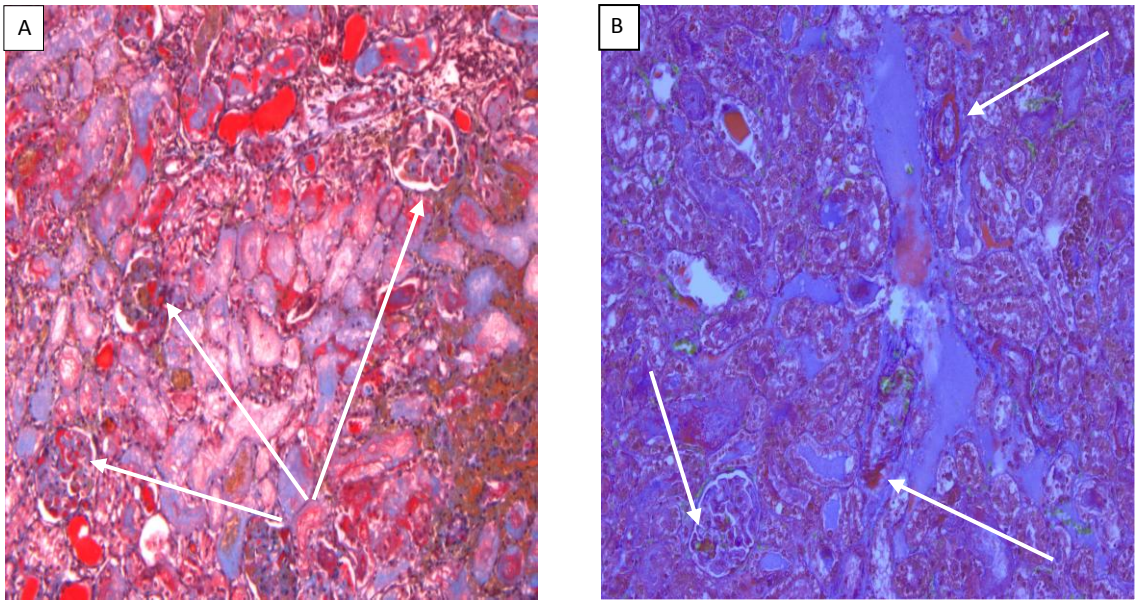


Figure 6.13: Sections of Soltran-perfused kidneys from recipients that received Rapamycin (n=4). A: Representative section of DA kidney allograft rejected at day 1 post-transplantation by a pre-sensitised Lewis recipient after Soltran/Rapamycin therapy showing widespread infarction, TMA and haemorrhage (MSB; x100). B: Extensive fibrinoid necrosis and TMA (MSB; x100).

6.9 Detection of Membrane Attack Complex (C5b-9) in rejected PTL004-treated Kidneys

Given the two-way interaction between complement and coagulation activation (reviewed in Chapter 1), my expectation was that anti-thrombin treatment, e.g. with PTL004, would inhibit complement activation (at the level of C3 or C5). To address the possible mechanisms by which PTL004 extended the graft survival, graft tissue was stained for the terminal complement activation product MAC (C5b-9). This would provide evidence of whether PTL004-mediated thrombin inhibition had an effect on complement activation, either by a direct action on the complement system or through preventing tissue damage mediated by the coagulation cascade. In this set of experiments, hyperimmune Lewis recipients were transplanted with Soltran-treated kidneys (n=2) or PTL004 pre-treated kidneys (n=2). In both cases the transplants were removed on day 1 or day 2 post-transplantation to enable direct comparison at the same time-points. Immunofluorescent staining of snap frozen tissue showed C5b-9 deposition in both groups and quantification was attempted, since it was clearly different. However, extreme infarction of the control tissue meant there were insufficient viable areas for comparison with the PTL004-treated tissue. Nonetheless, these experiments showed a striking contrast between the level of infarction in the Soltran-treated group and the level of protection in the PTL004-treated grafts. Although C5b-9 deposition was present in the treated group, it was focal rather than diffuse supporting a beneficial effect of PTL004 in the acute phase of accelerated rejection (Figure 6.14). The same pattern was found upon analysis of control and PTL004-treated tissue at 48 hours post-transplantation (Figure 6.15).

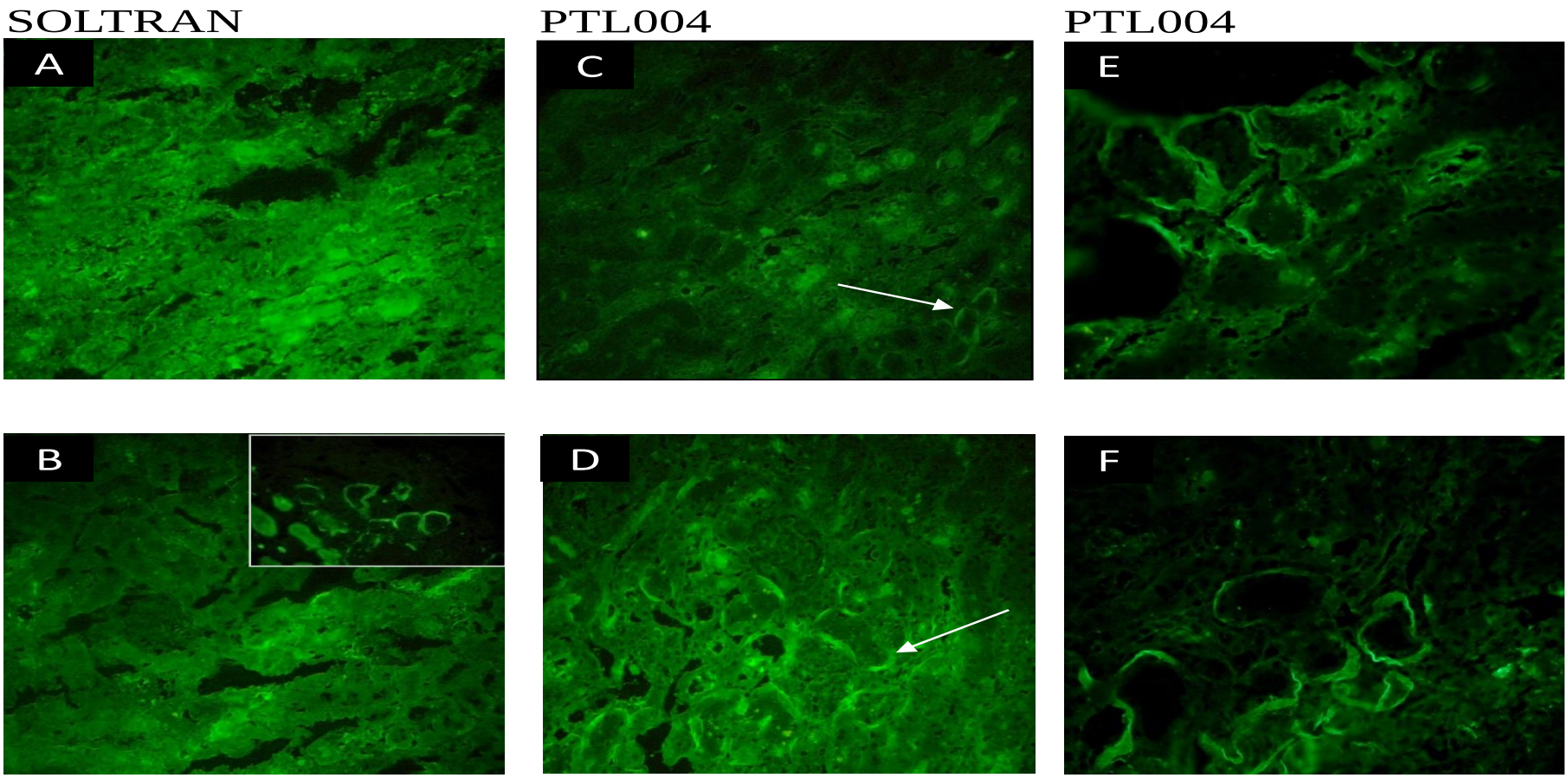


Figure 6.14: Donor kidneys were treated with 5 ml of 2μM PTL004 or Soltran alone and harvested at 24 hours post-transplantation. Kidneys were stained by immunofluorescence for C5b-9. A: Soltran-perfused allograft showing infracted tissue (x100). B: Infarction (x200), inset

showing linear deposition of C5b-9 on tubules in few viable areas (x400). C: PTL004-treated graft showing well preserved tissue and some positive C5b-9 staining (x100). D: Well preserved tubules stained for C5b-9 (x200). E, F: High power of tubules positively stained for C5b-9 (x400).

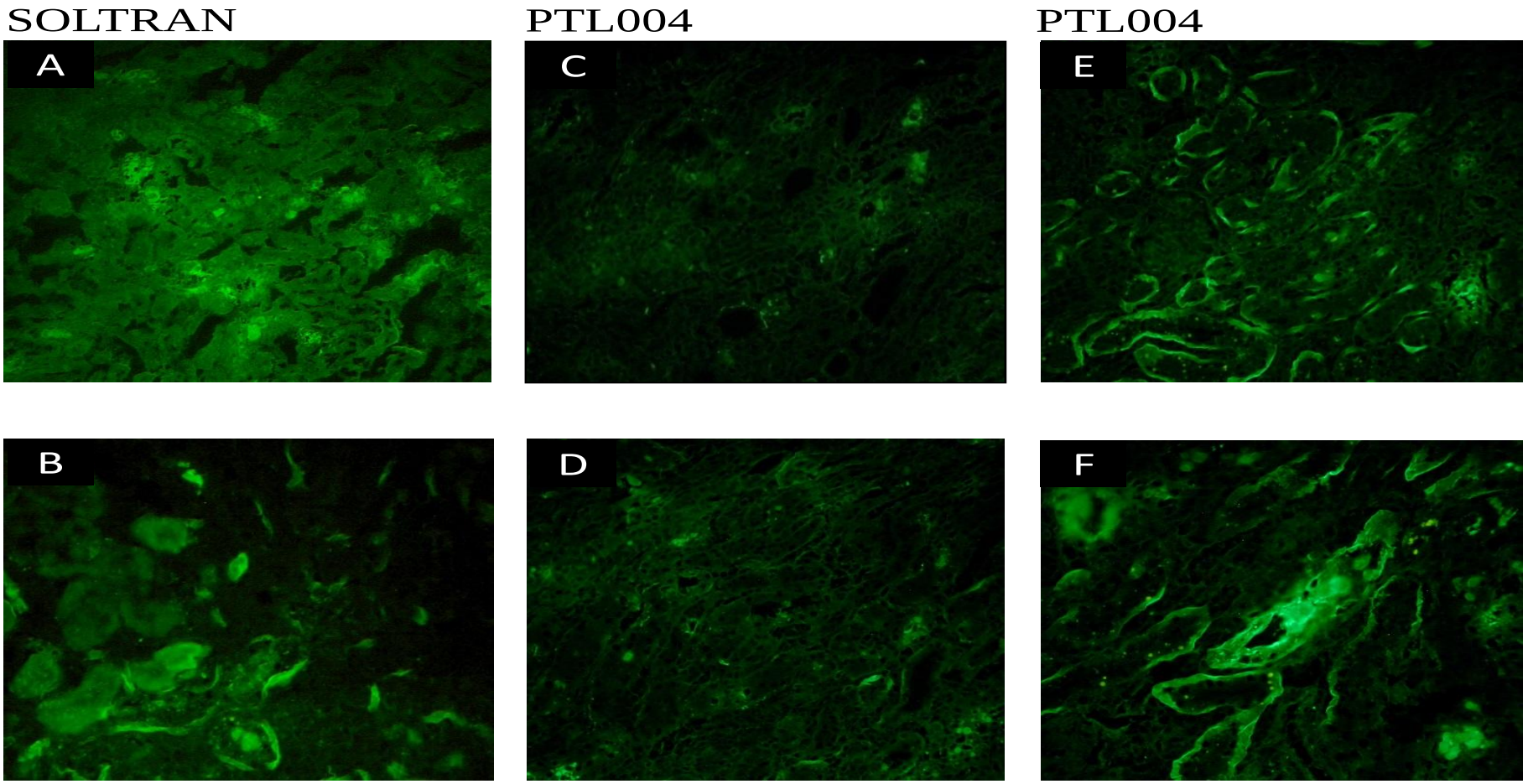


Figure 6.15: Donor kidneys treated with 5 ml of 2μM PTL004 or Soltran alone and removed from the pre-sensitised recipient at 48 hours post-transplantation. A: C5b-9 immunofluorescent staining of a Soltran-perfused allograft undergoing HAR showing severe infarction (x100). B: The

same kidney with infarction and some positive tubular staining for C5b-9 (x400). C: C5b-9 staining of a PTL004-perfused DA allograft undergoing AMR in a pre-sensitised Lewis recipient showing well preserved tissue and some positive C5b-9 staining (x100). D: Viable area of the same kidney with slight C5b-9 positive staining (x200). E: Intense staining of C5b-9 without necrosis (x200). F: High power of tubules positively stained for C5b-9 (x400).

6.10 Discussion

Following the encouraging results with PTL006 described in Chapter 5, the aim of the experiments described in the present chapter was to test the ability of a second thrombin inhibitor, PTL004, in reducing HAR in a rat allograft model. A second aim was to try a combination treatment comprising the thrombin inhibitor (targeting AMR) and standard immunosuppression (targeting cell-mediated rejection).

PTL004 and PTL006 are both thrombin inhibitors, but PTL004 has a novel membrane-inserting tail that is different to PTL006 (pegylated tail). The PTL004 has a double (bis) myristoyl group that creates the possibility of a more potent tail than that incorporated in PTL006 in terms of binding strength to the cell phospholipid bilayer membrane. Synthetic considerations and the observation that PTL006 was found to transfer ("painted") from loaded to unloaded cells more rapidly than PTL004 (Melchionna T, unpublished) led me to use PTL004 for further investigations.

Despite speculation that the bis-myristoyl tail of PTL004 may have a greater binding affinity than its predecessor molecule (PTL006), results generated using PTL004 were very similar to the ones produced using PTL006 in the previous chapter. One limitation to this observation is that PTL004 and PTL006 were never directly compared in a single experiment, and therefore exact conditions (including antibody status) may have differed. However, in broad terms the MST with both cytotopic antithrombins were similar. Intragraft perfusion of DA kidneys with PTL004 at 2 μM and transplantation into pre-sensitised Lewis recipients, significantly prolonged the graft survival (MST 5.1 days) compared with recipients of control Soltran perfused kidneys (MST 1.5 days). These results indicated that there is minimal influence of the tail on function of the inhibitor. Both tails mediate binding to the cell membrane. The level of protection of the renal vasculature from aggressive pro-inflammatory and pro-coagulant thrombin effects was no different, whether the hirulog peptide was attached to a pegylated or bis-myristoylated tail. Similarly to PTL006, the mechanisms by which PTL004 prolonged survival and protected from immediate renal dysfunction may be predicted to involve targeting of both complement-dependent and independent mechanisms (as discussed in detail in results chapter 5). In conclusion, PTL004 appeared to change the mode of rejection from hyperacute to acute, and histological analyses of graft tissue generated

after treatment with the thrombin inhibitor showed the consequences of mixed antibody and cellular immune responses.

An important aspect of this research was to investigate the suitability of these cytotopic agents (and their therapeutic protocol) for translation to the transplant clinic. For this reason, studies were extended to include standard immunosuppressive drugs to determine if the cell-mediated arm of rejection can be overcome by using combination therapy. This could maximise the chance of attaining graft acceptance against the most severe of immune barriers. Consequently, the sensitised recipients of PTL004-treated DA kidneys were treated with CsA before and after transplantation.

CsA failed to improve graft survival, despite the observation that in 50% of cases the cellular infiltration was abated by inclusion of CsA in the treatment protocol. The absence of improved outcome could have been due to the observed presence of tubular vacuolation and calcification which was unique to the CsA group, suggesting CsA toxicity. The combination of CsA toxicity with AMR could have aggravated tissue damage thereby negating any protective effect of PTL004. Why some recipients with signs of tubular injury succumbed immediately following transplantation while others survived longer is not clear but it could be due to variation in CsA pharmacodynamics and sensitivity in individual animals of the same species. Finally, it is possible that without the toxic effects, the longer surviving grafts of CsA-treated group could have had even more prolonged graft survival.

It is quite possible that the dose used in this model (10mg/kg/day), was causing severe nephrotoxicity, a feature that often has been associated with CsA during its use in acute experimental models of nephrotoxicity in the rat (Andoh TF et al., 1996 **315**). Moreover, the use of CsA has been associated with renal vascular thrombosis (Remuzzi G et al., 1989 **316**) and this may explain why in about half the animals the beneficial effect of PTL004 was no longer found. Indeed, recent research showed that CsA enhances the procoagulant properties of platelets by interaction with the platelet cell membrane (Tomasiak M et al., 2007 **317**). Spontaneous activation of platelets may result in enhanced thrombin generation, which ultimately causes expression of fibrinogen receptors (Fishman SJ et al., 1991 **318**). In other words, the susceptibility to graft thrombosis induced by antidonor antibody in this model could have been increased by CsA, obscuring the anticoagulant properties of PTL004 in a high proportion of

animals. Rapamycin inhibits the mammalian target of rapamycin (mTOR Complex 1) and therefore prevents proliferation and survival of lymphocytes (Ballou LM et al., 2008 **319)**.

The results with Rapamycin were more consistent than those with CsA. More specifically, 5 out of 6 had prolonged graft survival, however no graft survived beyond 10 days in either treatment group. A potential benefit of CsA treatment was that it appeared to prevent lymphocytic infiltration of the graph but these effects were limited by tubular toxicity and possibly arterial thrombosis, although tubular toxicity and possibly arterial thrombosis were observed. In contrast, recipients treated with Rapamycin showed no signs of toxicity but grafts had a substantial lymphocytic infiltration. Further adjustments in the dose of immunosuppressive agent -a decrease in the case of CsA and an increase for Rapamycin- could be productive in future experiments. This was not possible during the research described here due to limitations of time. Dose adjustment might be useful in the context of clinical translation, as it has been beneficial in man to prevent acute cell-mediated rejection.

Finally an experiment was carried out to find out if at least some of the therapeutic effects of PTL004 could have been due to an effect on complement activation, as well as on the coagulation cascade. Since thrombin is reported to directly cleave C5, I examined the formation of C5b-9 in transplanted kidneys that had been pre-treated with the anti-thrombin PTL004. The treated tissue was compared to that from Soltran-perfused kidneys. Residual staining was visualized as clear linear deposits on the tubular basement membranes, but the reduction seen compared with the Soltran-alone control group was not quantifiable in due to extensive tissue infarction in the control group. Nonetheless, this experiment was important since it showed that despite C5b-9 deposition in the PTL004-treated tissue, many areas were well preserved at 24 and 48 hours post transplantation. This was in contrast to the severely necrotic control tissue. Finally, these data support our hypothesis, that PTL004-mediated protection was taking place with ongoing complement activation, hence some MAC deposition.

Chapter 7

7.1 Conclusions

Transplants are powerful stimulants of alloantibody generation which are pivotal to graft rejection and loss. The risk for immediate transplant loss is highest in patients with preformed antibody (specific for ABO and/or HLA) against their donors. Nowadays, crossmatching techniques are used prior to transplantation to detect cross reactivity and avoid devastating HAR, however, memory generated due to pre-exposure still poses a barrier to successful long term transplantation.

The initial aim of the research presented in this thesis was to establish a rat transplant model in which a vascularized allogeneic kidney rejected in an aggressive and accelerated manner, resembling HAR in the clinic. Using a stringent rat strain combination, DA ($RT1^a$) to Lewis ($RT1^l$) with multiple donor skin transplant pre-sensitisation prior to kidney transplantation, a model akin to HAR was established. Using the universal criteria for the diagnosis of AMR, it was confirmed that the Lewis recipients had developed high titres of anti-DA specific alloantibody in their serum prior to kidney transplantation from the same donor strain. Aggressive humoral immune responses associated with high serum titres of alloantibody resulted in endothelial activation and subsequent initiation of the complement and coagulation cascades.

Using histological and immunopathological assessments, it was confirmed that coagulation- and complement- mediated injury was present in the graft removed from the pre-sensitised Lewis recipients. Acute tissue injury was evident, with extensive neutrophilic influx in the PTC and widespread necrosis. Most importantly, acute thrombotic microangiopathy (TMA) was seen in the presence of haemorrhage and fibrinoid necrosis. In addition, evidence for the involvement of complement in mediating tissue injury was revealed through C4d positive stained tissue in the rejected kidneys of pre-sensitised Lewis recipients.

Having established a model of HAR of renal allotransplants, an attempt was made at therapeutic intervention targeting one of the major rejection mechanisms involved in HAR, the complement cascade.

To target complement and the damaging effects of its split products, a well-characterized complement inhibitor, APT070 (Mirococept), was employed. APT070, a derivative of human soluble complement receptor 1 (CR1; CD35) derived from N-terminal 3 short consensus repeats and attached to a membrane inserting tail which allows it to efficiently bind to cells. This complement inhibitor retains the complement control mechanisms of CR1 (i.e decay acceleration and factor I cofactor activity). It has been successfully used in a variety of experimental models and humans (phase 1 trials for evaluation of safety, tolerability, pharmacokinetics and pharmacodynamiscs in 43 subjects in total) (study report ME0579, KCL, unpublished). Prior to proceeding to therapeutic intervention *in vivo*, in my model, experiments confirmed the capacity of APT070 to inhibit complement *in vitro.* Its ability to bind cells and its distribution *in vivo* after intrarenal delivery was also confirmed.

An important novel aspect of this model is that therapeutic intervention is administered directly to the kidney via the aorta and the renal artery thereby minimizing effects on systemic complement activity (Patel H et al., 2006 **291**). APT070 in Soltran was perfused intrarenally into DA donor kidneys which were then transplanted into hyperimmune Lewis recipients. The soluble non-tailed CR1 construct (APT154) was used as a control. APT070 was tested at two different doses (5 ml at 40 and 80 μg/ml) however, neither of the doses used, had any effect on recipient survival. The recipients of either APT070 or APT154 rejected their grafts by 48 hours post-transplantation (similar to that of recipients of Soltran-perfused control kidneys in chapter 3). A moderate improvement in renal function (BUN) and marginally better preserved graft tissue seen by histological examination was observed in the group that received kidneys treated with 80 μg/ml APT070. Nonetheless, the perfusion of DA kidneys with the APT070 complement inhibitor prior to transplantation into sensitised recipients did not extend graft survival.

HAR is the result of a well-orchestrated and complex immune response in which many mediators promote a local inflammatory environment thus promoting tissue injury. Having examined the possibility of targeting complement activation to improve graft outcome, targeting complement-independent mechanisms was then investigated.

In this model, the transplantation of DA kidneys into highly sensitised Lewis recipients resulted in graft histology showing extensive thrombosis. Thus, novel therapeutics

targeting thrombin (the central enzyme of the coagulation cascade) and their effect on graft outcome was investigated. In addition to targeting coagulation the inflammatory responses mediated by PAR receptors on a variety of cells could also be inhibited by an anti-thrombin strategy. The therapeutic reagent PTL006 was initially used. It was constructed following the same principle as APT070, that is, to be delivered directly in the target cell membrane.

The PTL006 thrombin inhibitor comprises the anti-coagulant hirulog-like peptide derived from the medicinal leech *Hirudo medicinalis*, coupled to a polyethelene glycol (PEG) tail. A major problem became evident during these studies, that is, excessive bleeding was occurring at the site of the arterial anastomosis (a condition which was amplified by the natural differences in size of the vessels between the donor and recipients rat strains). As a result, the intragraft perfusion protocol was modified to include two steps: 1) a ten-minute dwell-time following graft perfusion to allow longer for the reagent to bind cells followed by 2) an additional perfusion with 5 ml of Soltran solution to wash out any excess unbound material. These steps effectively eliminated uncontrolled bleeding during the transplantation procedure and enabled PTL006 (5 ml at 2 μM) to be perfused into the DA kidneys prior to successful transplantation into Lewis recipients. The anti-coagulant used under these conditions was able to significantly prolong graft survival (MST 4.6 days post-transplantation) in comparison to the Soltran-treated kidneys (MST 1.8 days post-transplantation). This was a pivotal finding since it changed the mode of rejection from rapid and extremely aggressive rejection to a slower acute response which might be more susceptible to therapeutic control. Progressive deterioration of renal function (BUN) occurred in the presence of both humoral and cellular rejection based on histological examination at the later time points of survival.

Hence, in this severe rejection model where the inhibition of complement activation had limited effects, blocking the coagulation cascade revealed that a degree of control was possible. A potential for therapeutic intervention therefore exists. Indeed, the inhibition of thrombin has a variety of effects, inhibiting both procoagulant and proinflammatory effects of alloantibody resulting in the protection of the graft. The therapeutic potential of combined treatment of DA kidneys perfused with PTL006/APT070 was explored. No significant additive effect was observed (MST 5.5 days post-transplantation) compared

to the anti-coagulant PTL006 alone, indicating once again the importance of the complement-independent alloantibody mechanism on the endothelium. These data question the role of complement activation in this model of HAR: complement activation might happen as result of it, but not cause it.

The successful extension of graft survival with PTL006 therapy required further investigation, with the aim to increase the life of the therapeutic on the vascular surface, which was suggested by immune staining to be less than 24 hours. For this, the thrombin inhibitor was changed to PTL004 (Thrombalexin). PTL006 has a PEGylated tail. As noted in Chapter 6, cell-bound PTL006 has the property of transferring rapidly to cells not bearing the agent whereas PTL004 appeared to be more stably bound (Melchionna T, unpublished data). PTL004 comprises the HLL peptide attached to a bis-myristoyl tail (an extra myristoyl group compared to APT070) thus potentially increasing its binding affinity to the phospholipid bilayer. DA kidneys perfused with 5 ml of 2 μM PTL004 were transplanted into hyperimmune Lewis recipients and similar survival was achieved compared to PTL006 (MST 5.1 days post-transplantation). These findings indicated that irrespective of the tail attached to the anti-thrombin HLL, this was the maximum survival which could be achieved in this model using a single intrarenal dose of the coagulation inhibitor.

The addition of a standard immunosuppressant was investigated to try and prevent the cell mediated component of rejection and further extend graft survival in this model, where rapid antibody-mediated responses have been dampened, at least during the acute phase of 24 hours post-transplantation. CsA with PTL004 was initially used, however tissue harvested from these recipients showed signs of nephrotoxicity, although for some of the animals prolongation of survival compared to recipients of CsA alone was achieved. As an alternative, Rapamycin was used in combination with PTL004-treated kidneys. Despite longer graft acceptance with this combination (MST 5.8 days post-transplantation) compared to the Rapamycin alone group (MST 1.5 days post-transplantation), tissue from the PTL004/Rapamycin group, was characterized by lymphocytic infiltration. This was an indication that the administration of Rapamycin in this model had not been completely effective in preventing lymphocyte migration into the grafts.

In summary, this thesis describes a novel therapeutic approach whereby coating the donor kidney with a membrane-adherent anti-thrombin reagent can slow the pace of rejection, which may then become more susceptible to control. In addition, it reveals that transplant surgery is feasible by reducing susceptibility for coagulation of the donor endothelium without inducing the risk of a major coagulation defect in the recipient. Efforts were made to measure systemic coagulation using a standard method and coagulometer (MC4 Plus Coagulometer, MERLIN medical, distributed by HART, UK). However data obtained were inconsistent and non reproducible. Alternative methods to detect the presence of the anticoagulant in the circulations are currently being developed. In a markedly pro-thrombotic environment, graft thrombosis/inflammation is significantly reduced probably for the duration of the cell surface activity of the tailed anticoagulant. The objective of this therapeutic strategy was to investigate the potential of localised therapy to prevent the deleterious effects of the alloresponse in pre-sensitised renal transplant recipients, without increasing the risk of systemic side effects. This study provided proof of concept for that strategy using a coagulation inhibitor, opening up the pathway for clinical translation.

7.2 Future Work

The work described in this thesis could be expanded in a variety of ways. Restrictions in time and difficulties in obtaining DA rats (due to breeding problems of the supplier) prevented some important experiments being carried out that could be addressed in the future.

The hyperimmune model described undeniably results in HAR. This provides a stringent test of the full potential of the therapeutic reagents as it is very severe transplant setting. Through crossmatching techniques between donors and recipients, HAR is not a phenomenon that often occurs in the clinic, therefore a less severe model of acute AMR may have been more appropriate in reflecting the situation of patients undergoing humoral rejection. However, the stringent model used was the ultimate test of efficacy. Nonetheless, this study is still highly relevant in view of the large number of pre-sensitised patients who would benefit from additional graft protection and who might otherwise be non-transplantable.

Attempts to target cellular rejection in this model were not effective using CsA or Rapamycin. This should be investigated further using a range of doses appropriate for each immunosuppressive reagent while treating the donor kidney cytotopically.

Finally, further investigations are required to dissect the mechanisms by which the anti-coagulants PTL006 and PTL004 protect the graft and change the type of rejection observed. For example, staining for expression of different endothelial proteins (such as vWf) in protected kidneys could give an explanation for this protective phenotype.

Preliminary data in this thesis showed that protection with the anticoagulant reagent takes place with ongoing complement activation (C5b-9). Nonetheless, this *in vivo* study could be used to extend data generated by Krisinger MJ et al. where it was identified that thrombin, acting like a convertase, cleaves C5 at a different site to that cleaved by C5 convertase to generate novel C5 products. *In vitro* assays showed that MAC formed by thrombin cleavage of C5, possessed greater activity than MAC generated by the standard C5 convertase (Krisinger MJ et al., 2012 **234**). Utilizing this model, the first *in vivo* study to establish the differentiation between formulation and origin of these different MAC could be done (provided an antibody is available in recognising the specific epitope at which thrombin cleaves). This may explain a

mechanism behind the interaction between the complement and coagulation cascades, and the protection by our cytotopic anticoagulants.

The work described here has formed the basis for a successful application to the Wellcome Trust for a Translation Award to advance Thrombalexin to first-in-man studies. The project has involved development chemistry to "fine-tune" the PTL004 structure (for example, removing the disulphide bridge which is reducible with compounds such as glutathione which are present in perfusion solutions such as University of Wisconsin solution). This project is well advanced and a selected compound is expected to be used in human studies within the next 18 months.

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http://kenpitts.net/bio/human_anat/kidney_nephron.gif

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